UNIVERSIDAD COMPLUTENSE DE MADRID
FACULTAD DE VETERINARIA

TESIS DOCTORAL

Desarrollo de vacunas recombinantes marcadoras multiserotipo frente al virus de la lengua azul

MEMORIA PARA OPTAR AL GRADO DE DOCTOR

PRESENTADA POR

Alejandro Marín López

Director

Francisco Javier Ortego Alonso

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UNIVERSIDAD COMPLUTENSE DE MADRID

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DESARROLLO DE VACUNAS RECOMBINANTES MARCADORAS MULTISEROTIPO FRENTE AL VIRUS DE LA LENGUA AZUL.

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Instituto Nacional de Investigación y tecnología agraria y alimentaria. Centro de Investigación en Sanidad Animal. INIA-CISA

Memoria presentada por Alejandro Marín López para optar al grado de Doctor por la Universidad Complutense de Madrid. Madrid, 2017.

El trabajo recogido en esta memoria ha sido realizado en el grupo de Inmunoprofilaxis de enfermedades virales transmitidas por vectores, en el Centro de Investigación en Sanidad Animal (CISA) perteneciente al Instituto Nacional de Investigación y tecnología agraria y alimentaria (INIA).

Francisco Javier Ortego Alonso, científico titular del centro de Investigación en Sanidad Animal (CISA-INIA) perteneciente al Instituto Nacional de Investigación y tecnología agraria y alimentaria (INIA).

INFORMA:

Que la presente tesis doctoral titulada "DESARROLLO DE VACUNAS RECOMBINANTES MARCADORAS MULTISEROTIPO FRENTE AL VIRUS DE LA LENGUA AZUL" que presenta D. Alejandro Marín López para optar al Grado de Doctor en Veterinaria, ha sido realizada en el grupo de Inmunoprofilaxis de enfermedades virales transmitidas por vectores del INIA-CISA bajo su dirección, y que reuniendo los requisitos exigidos y considerando que está concluida, autorizan su presentación para que pueda ser juzgada por el tribunal correspondiente.

Y para que así conste, firma el presente informe en Madrid a 22 de agosto 2017.

Fdo. Fco. Javier Ortego Alonso

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ABREVIATURAS

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ABREVIATURAS Y UNIDADES

A continuación se listan las abreviaturas y unidades más utilizadas en este trabajo. Para las abreviaturas, en algunos casos se indica su significado en inglés y en otros casos su traducción al castellano.

ºC Grados Celsius
aa Aminoácido
AHSV *African Horse Sickness virus*
APS Persulfato de amonio
ARV *Avian reovirus*
BE Brefeldina
BHK *Baby hamster kidney*
BSA Albúmina de suero bovino
BTV *Bluetongue virus*
CLP *Core-like particle*
CMV Citomegalovirus
ConA Concanavalina A
C-terminal Carboxilo terminal
CTL Linfocito T citotóxico
DAPI 4'-6-diamina-2'-fenilindol clorohidrato
DF-1 *Chicken embryo fibroblast*
DIVA Diferenciación entre animales infectados y vacunados
DL Dosis letal
DMEM Medio mínimo de Eagle modificado por Dulbecco
DNA Ácido desoxirribonucleico, ADN
dNTP Desoxirribonucleótido trifosfato
D.O. Densidad óptica
d.p.i Días post-infección
dsRNA RNA de doble cadena
EDTA ácido etilendiaminotetraacético
EHDV *Epizootic Hemorrhagic Disease virus*
ELISA Ensayo inmunoenzimático
EU Unión Europea
g Gramo
h Horas
H5 *High Five*
HIV Virus de la inmunodeficiencia humana
h.p.i. Horas post-infección
Hys Histidina
IF Inmunofluorescencia
IgG Inmunoglobulina G
IP Inmunoprecipitación
IPTG Isopropil-ß-D-1-tiogalactopiranósido
Kb Kilobases
kDa Kilo Dalton
LB Luria broth
log10 Logaritmo en base 10
M Molar
min Minutos

r r

ml Mililitro
MLV *Modified-live virus*
M.O.I. Multiplicidad de infección
mRNA RNA mensajero
MS Microesfera
MVA Virus vaccinia modificado cepa Ankara
N Normal
NSP Proteína no estructural
nt nucleótido.
N-terminal Amino terminal.
ORF Pauta abierta de lectura
OIE Organización mundial para la salud animal
PAGE Electroforesis en gel de poliacrilamida
PBS Tampón fosfato salino
PCR Reacción en cadena de la polimerasa
PF Paraformaldehído
p/p Peso/peso
p/v Peso/volumen
RNA Ácido ribonucleico, ARN
RNasa Ribonucleasa
rNTP Ribonucleótido trifosfato
RT Retrotranscripción
SDS Dodecilsulfato sódico
SFB Suero fetal bovino
TA Temperatura ambiente
TEMED Tretametil etilendiamina
TMB Tetrametil bencidina
TN Tris-NaCl
TNE Tris-NaCl-EDTA
TOV *Toggenburg orbivirus*
ug Microgramo
ufp Unidades formadoras de placas
ul Microlitro
UTR Región no traducida
V Voltio
Vero *African green monkey kidney*
VNT Test de neutralización viral
VV Virus vaccinia.
VLP Virus-like particle
VNT Test de neutralización viral
VP Proteína viral estructural
v/v Volumen/volumen
WB Western blot

RESUMEN EN INGLÉS

Bluetongue virus (BTV) belongs to the genus *Orbivirus* within the family *Reoviridae*. To date, 27 serotypes of BTV have been identified with two further putative BTV serotypes and other variants isolated so far. The BTV genome is composed of ten linear molecules of double-stranded RNA, which encode for seven structural and five non-structural proteins. BTV is the etiological agent of bluetongue (BT), a hemorrhagic disease of ruminants that is transmitted by the bite of female insects of the *Culicoides* genus.

The use of small laboratory animal models has allowed the study of the pathogenesis, immune response and vaccination strategies against BTV and other related orbiviruses in a quick, economic and reproducible manner. In this work, we provide an in-depth characterization of the histopathological features accompanying infection with BTV-4 of the well-characterized IFNAR(-/-) mouse model (deficient for type I IFN receptor). After BTV infection, a severe lymphoid depletion was observed in target tissues such as the thymus and the spleen, with a reduction of T cells in both tissues, as well as B cell populations in the spleen. We also observed an increment in the presence of monocytes/macrophages in these two organs and the lungs. Both caspase-3 and iNOS activities were also increased in spleen, indicating an exacerbated apoptotic process. Several pro-inflammatory cytokines were detected in these tissues, correlating with viral burdens. Finally, the levels of White Blood Cells (WBC), including lymphocytes, monocytes, neutrophils and platelets were altered in the infected animals. All these pathological alterations mimic those found in the natural hosts. Therefore, these data support the use of this animal model, which could contribute to facilitate the studies of viral pathogenesis and virus-host interactions and to aid in the development of new effective vaccines against BTV.

The IFNAR(-/-) mouse model was successfully employed to test vaccination strategies based on avian reovirus muNSMi microspheres tagged with the antigens VP2, VP7 and NS1 of BTV-4 or in a combination with recombinant vaccinia virus Ankara (MVA) expressing the same antigens. We analyzed the immune response elicited upon vaccination and the strength of these antigens as immunogens against BTV. Microspheres were able to induce neutralizing antibodies (Nabs) and CD4 T cell responses, and totally protected against a lethal challenge with an homologous infection with BTV-4, and partially against an heterologous infection with BTV-1. The combination of microspheres and rMVAs not only maintained the similar levels of Nabs but also induced strong CD8 T cell responses that are needed to confer protection against

multiple serotypes. Furthermore, animals immunized with this combined regimen survived challenge infections with both BTV-4 and BTV-1.

We next focused our studies on the non-structural protein NS1, because it is a highly conserved protein across all BTV serotypes and a major stimulator of cellular immune responses. Both factors are critical to achieve protection against multiple serotypes, as opposed to those elicited by surface antigens such as VP2, which are only able to confer protection against homologous infection through the induction of Nabs. In this work, we demonstrate that rMVA-NS1, used in a prime-boost regimen, was able to confer protection in IFNAR(-/-) mice against several BTV serotypes (BTV-1, BTV-4, BTV-8 and BTV-16), including one reassortant strain (BTV4-*Morocco,* BTV-4M). Vaccination resulted in the induction of a robust cytotoxic CD8 T cell response. Moreover, we demonstrate that the protective capacity of NS1 is located within the amino-terminus region, by generating a rMVA that expressed the first half of the NS1 aminoacid sequence (rMVA-NS1-Nt), that also induced a strong cytotoxic CD8 T cell response. Furthermore, we engineered rMVA-NS1-Nt with a deletion of a CD8 T epitope, named in this work p152 (rMVA-NS1-NtΔ152). When animals were immunized with this construct and challenged with a lethal dose of BTV-4, they succumbed to the infection, which was accompanied by a lack of CD8 T cell responses. These results confirmed that p152 is a necessary immunodominant peptide to induce protective immune responses against BTV. These data reveal the importance of the non-structural protein NS1 in CD8 T cell-mediated protection against multiple BTV serotypes when vectorized as a recombinant MVA vaccine and highlight the critical role of the response against the amino-terminus and the peptide 152.

In summary, our data highlight the benefits of the IFNAR(-/-) mouse model to address vaccine efficacy and BTV infection. This small animal model recapitulates several pathological features observed in infected natural hosts. Furthermore, these studies advance our knowledge in multiserotype vaccine development, including the use of combined vaccine platforms such as microspheres and rMVAs. They also demonstrate the importance of strong CD8 T cell-mediated responses against conserved, highly antigenic viral proteins such as NS1 as foundations of universal vaccine formulations.

RESUMEN EN ESPAÑOL

El virus de la lengua azul (*bluetongue virus*, BTV) pertenece al género *Orbivirus*, englobado en la familia *Reoviridae*. Hasta la fecha, se han descrito 27 serotipos, aunque se están estudiando nuevos aislados que podrían ser nuevos serotipos del virus. El genoma de BTV se compone de diez moléculas de RNA de doble cadena lineales, que codifican siete proteínas estructurales y cinco no estructurales. BTV es el agente etiológico de la enfermedad de la lengua azul (*bluetongue*, BT), una enfermedad hemorrágica que afecta a rumiantes y que es transmitida por la mordedura de las hembras del género *Culicoides.*

El uso de modelos animales pequeños de laboratorio ha posibilitado el estudio de la patogénesis, respuesta inmune y ensayos de vacunación frente a BTV y otros orbivirus relacionados, de una manera rápida, económica y reproducible. En este trabajo, analizamos en profundidad la caracterización de los aspectos histopatológicos que acompañan a la infección por BTV en el modelo de ratón IFNAR(-/-), ratón deficiente en el receptor del IFN tipo I. Tras la infección por BTV, se observa una fuerte depleción linfoide en los tejidos diana del virus como el timo o el bazo, con la consecuente reducción de las células T en ambos tejidos, así como una reducción de la población de células B en el bazo. También observamos un incremento en la presencia de monocitos/macrófagos en estos órganos y en pulmón. La actividad caspasa-3 así como iNOS, se incrementó en el bazo, indicando un proceso de apoptosis exacerbado. Se detectó un aumento de algunas citoquinas proinflamatorias en estos tejidos, que correlacionaba con la carga viral encontrada en ellos. Finalmente se observó una alteración en los niveles de células sanguíneas de la línea blanca, como linfocitos, monocitos, neutrófilos y plaquetas en los animales infectados. Todas estas alteraciones patológicas mimetizan a las observadas en el hospedador natural, por lo que estos resultados apoyan el uso de este modelo animal a la hora de avanzar en el estudio de la patogénesis, interacción virus-hospedador así como facilitar el desarrollo de nuevas vacunas efectivas frente a BTV.

El modelo de ratón IFNAR(-/-) ha sido empleado en este trabajo para el estudio de nuevas estrategias de vacunación basadas en microesferas generadas con la fracción mínima de la proteína muNS del reovirus aviar (ARV) que incorporan los antígenos VP2, VP7 y NS1 de BTV-4, o en microesferas combinadas con el vector viral vacunal recombinante MVA (virus vaccinia Ankara) expresando dichos antígenos. Analizamos la respuesta inmune inducida tras la vacunación y la potencia de estos antígenos como inmunógenos frente a BTV. Las

microesferas fueron capaces de inducir un nivel elevado de anticuerpos neutralizantes (Nabs) así como una potente respuesta celular T CD4, quedando los animales totalmente protegidos frente a la infección homóloga con una dosis letal de BTV-4, y parcialmente protegidos frente al desafío heterólogo con BTV-1. La combinación de microesferas y rMVAs no solo mantuvo niveles similares de Nabs sino que también estimuló una fuerte respuesta celular T CD8, necesaria para controlar la infección frente a múltiples serotipos. Además, los animales inmunizados siguiendo esta estrategia combinada sobrevivieron tanto al desafío homólogo con BTV-4 como al heterólogo con BTV-1.

Por último, nos centramos en el estudio de la proteína no estructural NS1, debido a que es la proteína más conservada entre todos los serotipos de BTV y es el principal estimulador de la respuesta inmune celular. Ambos factores son críticos para conseguir protección frente a múltiples serotipos, a diferencia del tipo de respuesta conferida por los antígenos de superficie del virión como la proteína VP2 que solo capaz de conferir protección frente a infecciones homólogas a través de la inducción de Nabs. En este trabajo hemos demostrado que el vector viral rMVA-NS1, siguiendo una estrategia *prime-boost* de vacunación, es capaz de conferir protección en ratones IFNAR(-/-) frente a múltiples serotipos de BTV (BTV-1, BTV-4, BTV-8 y BTV-16), incluyendo una cepa surgida de forma natural por reorganización génica entre virus de serotipo 4 y 1 (BTV-4 *Morocco,* BTV-4M).

La vacunación con rMVA-NS1 activó una fuerte respuesta T CD8 citotóxica. Además, generando un rMVA que expresa la primera mitad de la secuencia de aminoácidos de NS1 (rMVA-NS1-Nt) demostramos que esta capacidad protectora de NS1 se encuentra en la región amino-terminal, activando la misma respuesta T CD8 citotóxica que la proteína entera. Por otra parte generamos un vector (rMVA-NS1-NtΔ152) con la deleción de un epítopo T CD8, denominado en este estudio p152. Los animales inmunizados con esta construcción no sobrevivieron a la infección con una dosis letal de BTV-4, acompañada por la pérdida de la respuesta T CD8. Estos resultados confirman que p152 es un epítopo inmunodominante necesario para inducir una respuesta inmune protectora frente a BTV. Estos datos revelan la importancia de la proteína no estructural NS1 en la protección mediada por la respuesta T CD8 frente a múltiples serotipos de BTV cuando es vehiculizada como una vacuna MVA recombinante y pone de manifiesto el papel esencial de la respuesta inmune CD8 frente a la región amino-terminal y el péptido 152.

Finalmente, todos estos datos destacan las ventajas del empleo del modelo de ratón IFNAR(-/-) en los estudios de eficacia de vacunas y en los estudios de patogénesis por infección

de BTV. Este animal de laboratorio mimetiza muchos de los aspectos observados en la infección del hospedador natural. Además este trabajo ha permitido avanzar en el desarrollo de vacunas multiserotipo, incluyendo el uso de estrategias de inmunización combinadas como las aquí presentadas basadas en microesferas y rMVAs. Estos estudios demuestran la importancia de la respuesta inmune mediada por células CD8+ frente a proteínas tan conservadas y antigénicas como la proteína no estructural NS1 como base para un candidato de vacuna universal frente a BTV.

INTRODUCCIÓN

El virus de la lengua azul, en inglés *bluetongue virus* (BTV), es el agente causal de la enfermedad de la lengua azul (LA), también conocida como fiebre catarral ovina. BTV es el virus prototipo del género *Orbivirus*, perteneciente a la familia *Reoviridae,* familia con una gran importancia tanto en salud veterinaria como humana (Mertens, 2005). La lengua azul es una enfermedad hemorrágica que afecta tanto a rumiantes silvestres como domésticos y también a camélidos (Verwoerd et al., 1972). Hasta la fecha han sido descritos 27 serotipos diferentes de BTV, de los cuales, tres de ellos han sido identificados recientemente, los serotipos 25, 26 y 27 (Hofmann et al., 2008; Jenckel et al., 2015; Maan et al., 2011) y tres posibles nuevos serotipos están siendo analizados: BTV-28 detectado en vacunas contra Capripox en Oriente Medio (Kyriaki Nomikou, comunicación personal), BTV-29, aislado de una alpaca en Sudáfrica (Maan et al., 2015; Sun et al., 2016; Wright, 2014) y un tercero aislado en Córcega en cabras (Savini et al., 2017).

1. Clasificación taxonómica del virus de la lengua azul.

1.1. *Familia Reoviridae.*

La familia Reoviridae engloba un grupo de virus que comparten una serie de propiedades morfológicas y genéticas comunes como: poseer un genoma lineal de RNA de doble cadena (dsRNA) fragmentado en 10-12 segmentos genómicos, contener dos o tres capas proteicas concéntricas, carecer de una envoltura lipídica, presentar una estructura icosahédrica con un diámetro de 70 a 85 nm y replicar en el citoplasma de la célula infectada (Norkin, 2010).

El prefijo reo proviene del término "*Respiratory Enteric Orphan*" y fue propuesto para agrupar una serie de virus que fueron aislados de los tractos respiratorio e intestinal de individuos aparentemente sanos (Sabin, 1959). De ahí el término "*Orphan*" cuyo significado en inglés es huérfano y hace referencia al desconocimiento de alguna enfermedad a la que poder asociar el virus. Previamente a esto, esos virus habían sido incluidos dentro de la familia de los *echovirus*. Los virus que pertenecen a la familia *Reoviridae* están muy extendidos en la naturaleza e infectan a un amplio espectro de hospedadores como plantas, hongos, insectos, peces, reptiles, aves y mamíferos, incluyendo humanos (http://viralzone.expasy.org/all_by_species/104.html). En los organismos infectados, los reovirus pueden afectar desde una manera asintomática hasta causar lesiones fatales. Basándose en las características estructurales de los viriones, en el número de segmentos génicos y en las estrategias del ciclo replicativo, se han descrito quince géneros para agrupar a

los miembros de esta familia viral; *Aquareovirus, Coltivirus, Cypovirus, Dinovernavirus, Fijivirus, Idnoreovirus, Mycoreovirus, Orthoreovirus, Oryzavirus, Cardoreovirus, Mimoreovirus, Orbivirus, Phytoreovirus, Rotavirus y Seadornavirus* (Mertens, 2005).

1.2. *Género Orbivirus.*

El nombre de este género proviene del término en latín orbis, que significa anillo o círculo, y hace referencia a la capa proteica interior de los orbivirus que está formada por 32 capsómeros en forma de anillo (Gorman, 1985). El genoma de los orbivirus está formado por 10 segmentos génicos de dsRNA; tradicionalmente se pensó que todos eran monocistrónicos, pero en los últimos años se ha podido comprobar que en el caso de ciertos virus algunos segmentos codifican más de una proteína, como es el caso de los segmentos 9 y 10 en BTV o 9 y 11 en el caso de algunos rotavirus. Las regiones terminales 5' y 3' están conservadas (5'-GUUAAA....ACUUAC-3') en cada segmento de dsRNA de los orbivirus y tienen complementariedad invertida (Roy, 2001). Los miembros del género Orbivirus son capaces de infectar a un gran número de especies de vertebrados e insectos. Además de BTV, hay otras 21 especies de este género que se transmiten por medio de artrópodos, lo que se conoce con el término de arbovirus (*Arthropod Borne Viruses*), con excepciones, como el serotipo 26 de BTV, para el cual se ha demostrado que existe una transmisión aérea directa (Batten et al., 2014). Las especies de Orbivirus son causantes de un gran número de enfermedades emergentes, algunas de las cuales suponen un gran riesgo para la salud animal en Europa como BTV y el virus de la peste equina africana ("*African horse sickness virus*" o AHSV). Otras especies de orbivirus con gran potencial de riesgo son el virus de la enfermedad hemorrágica epizoótica ("*Epizootic Hemorragic Disease virus*" o EHDV), el virus de la encefalitis equina ("*Equine encephalosis virus*" o EEV) y el virus de la peste equina peruana ("*Peruvian horse sicknes svirus*"o PHSV) (Tabla 1). Se han descubierto nuevos orbivirus, como el recientemente identificado como *Tibet Orbivirus* (TBOV), descubierto en Tibet, China, y aislado del mosquito *Anopheles maculatus*, cuyo hospedador hasta la fecha se desconoce (Li et al., 2014) y otro denominado *Muko virus*, aislado de garrapatas del género *ixodes* en Japón, cuyos hospedadores naturales son las aves migratorias (Ejiri et al., 2015). Otra especie recientemente descubierta es el llamado *Parry's Lagoon virus*, PLV, hallado en Australia y aislado de las especies de mosquitos *Culex annulirostris, Culex pullus, Mansonia uniformis* y *Aedes normanensis.* A pesar de presentar una moderada-alta identidad en cuanto a la secuencia de aminoácidos de sus proteínas con el virus Corriparta (CORV), este nuevo virus no replica en las

células de vertebrado que sí son permisivas a la infección con CORV, lo que podría indicar que el virus solo tiene ciclo de replicación en el mosquito (Harrison et al., 2016).

Tabla 1. Especies pertenecientes al género *Orbivirus*. Esta tabla muestra las 22 especies identificadas dentro del género orbivirus, así como los serotipos identificados, vector transmisor y hospedador natural. http://www.ictvonline.org/virusTaxonomy.asp?src=NCBI&ictv_id=20074304

Especies	Serotipos	Vector	Hospedador
Peste equina (AHSV)	AHSV 1-9	Culicoides	Équidos, perros, elefantes, ganado, camellos, cabras, ovejas, carnívoros depredadores
Lengua azul (BTV)	BTV 1-27	Culicoides	Oveja, cabras, elefantes, ganado, rumiantes (tanto domésticos como salvajes), carnívoros depredadores
Changuinola	Almeirin, Altamira, Caninde, Changuinola, Gurupi, Iratuia, Jamanxi, Janti, Monte Dorado, Ourem, Purus, Saraca	Flebotomos y mosquito	Humanos, roedores, perezosos
Chenuda	Baku, Chenuda, Essaouria, Huncho, Kala Iris, Mono Lake, Sixgun City	Garrapata	Aves marinas
Chobar Gorge	ChobarGorge, Fomede	Garrapata	Murciélagos
Corriparta	Acado, Corriparta 1-4, Jacareacanga	Flebotomos y mosquitos	Humanos y roedores
Enfermedad hemorrágicaepizoótica (EDHV)	EHDV 1-9, Ibaraki	Culicoides	Ganado, ovejas, ciervos, camellos, llamas, rumiantes salvajes, marsupiales
Encefalitis equina (EEV)	EEV 1-7	Culicoides	Équidos
Eubanangee	Eubanangee, Ngoupe, Pata, Tilgerry	Culicoides, Anofelinos y Mosquitos	Desconocido

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Great Island	Above Maiden, Arbroath, Bauline, Broadhaven, Cape Wrath, Colony, Colony B, Ellidaey, Foula, Great Island, Great Saltee Island, Grimsey, Inner Frame, Kermerovo, Kenai, Kharagysh, Lipovnik, Lundy, Maiden, Mill Door, Mykines, NothClett, North End, Nugget, Okhotskiy, Poovoot, Rost Island, Saint Abb, Shiant Island, Thormodseyjarlettur, Tillamook, Tindholmur, Tribec, Vearoy, Wexford, Yaquina	Garrapata	Aves marinas, roedores y humanos
Ieri	Ieri, Gomoko, Arkonam	Mosquitos	Aves
Lebombo	Lebombo	Mosquitos	Humanos y roedores
Orungo	ORU 1-4	Mosquitos	Humanos, camellos, ganado, cabras, ovejas y monos
Palyam	Abadina, Bunyip Creek, CSIRO village, Daguilar, Gweru, Kasba, Kindia, Marrakai, Marondera, Nyabira, Palyam, Petevo, Vellore	Culicoides	Ganado y ovejas
Peste equina peruana (PHSV)	PHSV-1	Mosquitos	Caballos
Rio St.Croix	Rio St. Croix	Garrapata	Desconocidos
Umatilla	Llano seco, Minnal, Netivot, Umatilla	Mosquitos	Aves y Humanos

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Wad Medani	Seletar, Wad Medani	Boofilos, Rhipicefalus, Hyalomma, Argas	Oveja, perro, cerdo, burro, roedor, camellos, humanos.
Wallal	Mudjinbarry, Wallal	Culicoides	Canguros y Wallabies
Warrengo	Mitcher, Warrengo	Culicoides	Canguros y Wallabies
Wongorr	ParooRiver, Picola, Wongorr 1-6	Culicoides, Anofelinos y Mosquitos	Ganado y macropódidos
Yunnan Orbivirus (YUOV)		*Mosquito Culex tritaaeniorhynchus*	Desconocido

2. Características moleculares y biológicas del virus de la lengua azul.

2.1. *El genoma de BTV.*

El virus de la lengua azul posee un genoma segementado formado por diez moléculas de RNA de doble cadena, los cuales comprenden 19.200 pares de bases (pb). Tradicionalmente se ha pensado que cada uno de estos segmentos era monocistrónico, codificando una única proteína. Sin embargo, en los últimos años se ha comprobado que, al menos, dos segmentos presentan dos marcos abiertos de lectura diferentes (ORF) (Belhouchet et al., 2011; Ratinier et al., 2011; Stewart et al., 2015). Los diez segmentos que conforman el genoma de BTV están numerados del 1 al 10, cada uno de los cuales codifica un único polipéptido, con excepción de los dos segmentos más pequeños (9 y 10), que codifican las proteínas VP6 y NS4, y NS3/NS3A y NS5, respectivamente (Belhouchet et al., 2011; Mertens, Brown, and Sangar, 1984; Ratinier et al., 2011; Stewart et al., 2015). En la Tabla 2 se detallan todas las proteínas conocidas de BTV, los segmentos que las codifican y las funciones conocidas de cada una.

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Tabla 2. En esta tabla se muestran cada una de las proteínas de BTV, su localizaión y función. *Adaptado de Mertens et al., 1984* (http://www.reoviridae.org/dsRNA_virus_proteins/BTV.htm).

Segmentos genómicos y proteínas codificadas de BTV				
Segmento Genómico	***bp***	***Proteína/s (aa)***	***Localización***	***Función***
1	3954	VP1 (1302)	Interior del core	RNA Polimerasa RNA dependiente
2	2926	VP2 (956)	Cápside externa	Proteína estructural específica de serotipo Hemaglutinina Entrada en la célula
3	2772	VP3 (901)	core	Proteína estructural, forma el soporte para el anclaje de los trímeros VP7 Necesaria para el ensamblaje del virión
4	2011	VP4 (644)	Interiordel core	Enzima que cataliza la adición de residuos cap a los mRNA. guanilitransferasa/ metiltransferasa
5	1770	NS1 (552)	No estructural	Forma túbulos en el citoplasma celular Función desconocida
6	1639	VP5 (526)	Cápsideexterna	Proteína estructural Permeabilización celular
7	1156	VP7 (349)	Superficie del core	Proteína estructural. Posible función en la entrada celular en el vector
8	1123	NS2 (357)	No estructural	Forma los cuerpos de inclusión, fosfoproteína que une ssRNA
9	2046 182-418	VP6 (329) NS4 (77-79)	Interior del core No estructural	Une ssRNA&dsRNA, actividad helicasa Posible función protectora ante degradación de DNA Antagonista de interferon y determinante de virulencia
10	822 59-709 108-287	NS3/NS3A (229/226) NS5 (80)	No estructural	Glicoproteína, participa en la salida del virión Puede participar en la modulación de la transcripción celular Puede tener funciones específicas en el nucleolo

Estudios recientes han descrito cómo el virus de la lengua azul empaqueta su genoma durante la infección, identificándose motivos estructurales en los extremos 3' y 5' en el segmento 4 de BTV-9 y en el segmento 8 de BTV-1, esenciales para llevar a cabo esta función, ya que estos motivos por sí mismos son capaces de empaquetar secuencias no relacionadas con el genoma del virus (Burkhardt et al., 2014). Además, ensayos de interacción RNA-RNA *in vitro* sugieren que el empaquetamiento se inicia a partir de la región no traducida (UTR) del segmento más pequeño, el segmento 10, el cual interacciona y recluta los segmentos de tamaño mediano y después los más grandes (Roy, 2017; Sung and Roy, 2014). Ensayos en los que se ha llevado a cabo mutaciones puntuales en los UTR 5' y 3' del segmento 10 ponen de manifiesto la inviabilidad del virus, debido a que los cambios en la estructura secundaria del RNA producidos por dichas mutaciones no permiten el correcto empaquetamiento de los segmentos (Boyce and McCrae, 2015). Esta organización estructural de los segmentos de dsRNA viral puede además ayudar a la transcripción de la información genómica ya que la transcriptasa será capaz de realizar su función eficazmente, evitando la aparición de posibles estructuras secundarias que dificulten su actividad (Diprose et al., 2001).

2.2. *Estructura del virión.*

La estructura madura del virión se compone de tres capas protéicas concéntricas (capa externa, intermedia e interna) sin envoltura. Presenta una geometría icosahédrica con aproximadamente 80 nm de diámetro (Hewat, Booth, and Roy, 1992). La capa interna define el core, donde se encuentra el genoma del virus (Gouet et al., 1999).

El virión de BTV está formado por siete proteínas estructurales (VP1 a VP7) (Figura 1). Durante el ciclo de infección se producen 5 proteínas no estructurales, NS1, NS2, NS3/ NS3A, NS4 and NS5 que no forman parte de la estructura del virión (Belhouchet et al., 2011; Ratinier et al., 2011; Stewart et al., 2015).

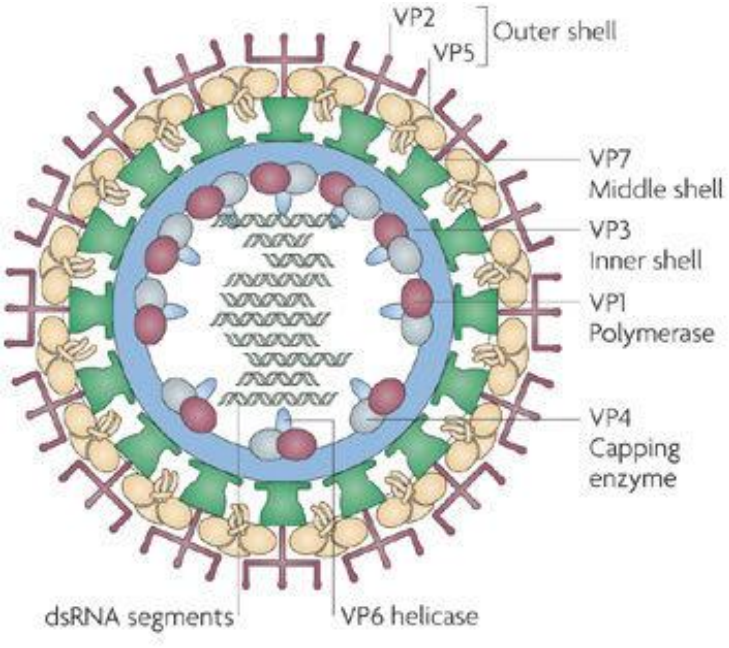


Figura 1. Morfología de BTV. Tres capas concéntricas envuelven el genoma y las proteínas que conforman el complejo de replicación del virus (Roy, Boyce, and Noad, 2009).

2.2.1. Proteínas de la capa externa: VP2 y VP5.

Las proteínas de la capa externa VP2 y VP5 rodean el core, formado por la proteína VP3, y la capa intermedia formada por la proteína VP7. Está constituida por 60 trímeros (180 copias) de VP2, la cual presenta una estructura en forma de triskelion que sobresale de la capa externa, siendo la más expuesta en el virión maduro (Hyatt, Zhao, and Roy, 1993) y 120 trímeros (360 copias) de la proteína VP5 (Burroughs et al., 1995; Hewat, Booth, and Roy, 1992). La proteína VP2 es la responsable de la unión al receptor celular (Huismans and Erasmus, 1981), tiene capacidad hemaglutinante (Cowley and Gorman, 1987) y es inductora de anticuerpos neutralizantes específicos de serotipo (Eaton and Crameri, 1989; Hassan and Roy, 1999; Marshall and Roy, 1990; Roy, 1992; Urakawa et al., 1994). Estudios en los que se ha eliminado la proteína VP2 del virión muestran la incapacidad del virus para unirse a células BHK-21, lo que sugiere que esta proteína es la responsable de la unión entre el virus y la célula (Huismans and Erasmus, 1981). El receptor celular que BTV utiliza para entrar en las células del hospedador no se encuentra del todo caracterizado. Se han identificado posibles receptores que interaccionen con los viriones en la superficie celular. Glicoproteinas como la glicoforina A, un componente sialoglicoproteico de los eritrocitos, presenta una fuerte afinidad por VP2.

Dicha interacción puede estar implicada en el anclaje de BTV a los eritrocitos (Eaton and Crameri, 1989; Hassan and Roy, 1999). Además, hay cierta evidencia de que las moléculas de heparan-sulfato de la superficie de la membrana celular pueden mediar en la unión con el virión (Mecham and McHolland, 2010). Además, la proteína VP2 recombinante y la glicoforina A pueden inhibir la unión de BTV a células susceptibles, sugiriendo que el mecanismo receptor de BTV consiste en la interacción de VP2 con una glicoproteína de la superficie celular (Hassan and Roy, 1999). Dentro de la célula, VP2 se asocia a vimentina, lo que permite la localización sub-celular de la proteína y la interacción de la partícula madura de BTV con los filamentos intermedios. También hay estudios que sugieren que esta proteína está involucrada en la salida del virus (Bhattacharya, Noad, and Roy, 2007). La disrupción de la interacción VP2/vimentina por inhibidores farmacológicos conlleva el bloqueo de la liberación del virus (Bhattacharya, Noad, and Roy, 2007). VP2 es el principal determinante del serotipo de BTV, y con una implicación menor la proteína VP5 (Mertens, Burroughs, and Anderson, 1987), por ello se utiliza a menudo en el diagnóstico de nuevos brotes de BTV (Mertens et al., 2007). VP2 es la proteína menos conservada del virión, siendo diana de los anticuerpos neutralizantes generados por el sistema inmune del hospedador, por lo que VP2 es la responsable de definir cada uno de los serotipos de BTV (White and Eaton, 1990). La comparación filogenética de VP2 entre las 27 cepas de referencia ha mostrado una perfecta correlación entre la variación de secuencia del segmento genómico 2 (Seg-2), que codifica la VP2, y el serotipo de BTV. Las secuencias del seg-2 de los 27 serotipos de referencia de BTV se pueden agrupar en diez linajes con distinta evolución, identificados como nucleotipos A–J, que son nodos de divergencia entre diferentes serotipos durante su evolución (Pavuluri Panduranga Rao, 2017). Las secuencias de nucleótidos correspondientes a VP2 varían desde un 29% (BTV-8 y BTV-18) a un 59% (BTV-16 y BTV-22) entre los distintos serotipos. A pesar de toda la variabilidad de secuencia, algunas características de la proteína VP2 parecen estar conservadas a través de los serotipos, incluyendo el perfil hidrofóbico, distribución de la carga y la posición de ciertos residuos de cisteína (Maan et al., 2007).

Otra agrupación es la que se ha realizado teniendo en cuenta mutaciones puntuales en el genoma de BTV debido a errores de la RNA polimerasa del virus. Algunos de estos cambios se acaban estableciendo en la población local de un virus, adquiriendo dichas poblaciones diferentes mutaciones puntuales, algunas de las cuales pueden conferir una ventaja adaptativa en cuanto a la transmisión y la supervivencia del virus (Maan, 2009b).

La proteína VP5 está más conservada que VP2, mostrando cierto grado de variación reflejado en el origen geográfico (Singh et al., 2004). Los trímeros de VP5 forman los motivos

globulares de la cápsida exterior de la partícula viral de BTV (Nason et al., 2004). La estructura secundaria predicha para esta proteína indica que VP5 presenta un dominio globular en su región C-terminal y un dominio *coiled-coil* unido por una región bisagra en su región N-terminal. Éstas hélices descritas en el extremo N-terminal de la proteína hacen que comparta una estructura muy similar con las proteínas de fusión de clase I de los virus con envoltura (Forzan, Wirblich, and Roy, 2004; Hassan et al., 2001; Nason et al., 2004), por lo que se cree que VP5 tiene un papel esencial en la entrada del virus a través de membranas celulares, una actividad de fusión de membranas que ayudaría al virión durante su entrada en la célula así como en la liberación de las partículas virales desde los compartimentos endosomales hacia el citoplasma (Hassan et al., 2001) previa activación a pH ácido, lo que provoca un cambio conformacional que permite la fusión de la membrana y que conduce a la formación de sincitios (Forzan, Wirblich, and Roy, 2004). Se ha comprobado que la region N-terminal de VP5 es esencial para que se lleve a cabo la fusión de membranas tanto en células de mamífero como de *Culicoides* (Hassan et al., 2001) y también que VP5 se asocia a las balsas lipídicas (*lipidrafts*) presentes en la membranas celulares a través de las proteínas SNARE (Bhattacharya and Roy, 2008).

2.2.2 Proteínas del core: VP3 y VP7.

La proteína VP3 forma homodímeros, que a su vez forman decámeros, los cuales constituyen el core de BTV (120 copias de VP3), presentando una estructura icosahédrica (Grimes et al., 1998). VP3 y VP7 contienen determinantes antigénicos específicos de serotipo que definen distintos grupos filogenéticos (Anthony et al., 2007). VP3 está altamente conservada y junto con VP7, juega un papel importante en la integridad estructural y el ensamblaje del core viral. Mutantes de la proteína VP3 que presentan deleciones en el dominio de dimerización no son capaces de iniciar la formación de los cores, aun siendo capaces de formar los dímeros y decámeros (Kar, Ghosh, and Roy, 2004). Estudios basados en cristalografía de rayos X han identificado muescas en la cara interna del core formado por VP3, las cuales se cree que pueden estar involucradas en la organización del genoma del virus (Mertens and Diprose, 2004) y que se asocian a las enzimas del complejo de replicación. Más concretamente, se ha demostrado que las proteínas VP1 y VP4 se asocian a los decámeros de VP3 para iniciar el ensamblaje del core (Le Blois et al., 1992). La capa intermedia del virion la componen 260 moléculas de trímeros de VP7, justo por debajo del triskelion de VP2. La parte más alta de VP7 interacciona con VP2, mientras que las regiones laterales lo hacen con los dominios globulares de la proteína VP5 (Hewat et al., 1992; Nason et al., 2004). Aunque los cores son poco o nada infecciosos en diferentes células de mamífero, son al menos 100 veces

más infecciosos que el virión en los *Culicoides* adultos o en la línea celular de *Culicoides* (células KC) (Mellor, 1990; Mertens et al., 1996). VP7 puede mediar el anclaje y la entrada en células de insecto en ausencia de VP2 o VP5 (Tan et al., 2001). El complejo VP3/VP7 protege el genoma viral compuesto por dsRNA de la vigilancia intracelular, previniendo la activación de la producción de interferón tipo I (IFN α/β) vía sensores citoplásmicos como las helicasas citosólicas, o interacciones con mecanismos de corte y silenciamiento del RNA (Gouet et al., 1999; Jacobs and Langland, 1996).

2.2.3. Proteínas minoritarias del core: VP1, VP4 y VP6 (complejo de transcripción).

Las proteínas que forman parte del complejo de replicación son VP1 (polimerasa), VP4 (enzima de capping de mensajeros) and VP6 (helicasa). La proteína VP1 está presente en un radio molar bajo, aproximadamente 12 copias por partícula, dentro del virión (Huismans and Van Dijk, 1990; Stuart et al., 1998). VP1 puede extender la síntesis de RNA a partir de oligonucleótidos y actuar como replicasa que sintetiza dsRNA a partir de un molde de RNA de cadena positiva (Boyce et al., 2004). La actividad óptima de VP1 tiene lugar entre 27ºC y 37ºC, lo que permite una replicación eficiente tanto en células de insecto como de mamífero. A los mRNAs tempranos se les añade un residuo "cap" (metilguanosina unida al primer nucleósido) que estabiliza el mRNA y permite una traducción eficiente. En células, este proceso requiere la acción de cuatro actividades enzimáticas distintas (dos reacciones transmetilasa, una reacción guanililtransferasa y otra nucleótido fosfohidrolasa). En BTV, esas cuatro reacciones son catalizadas por una única proteína, la VP4, cuya estructura cristalina muestra una arquitectura modular alargada que proporciona un soporte para el ensamblaje de sitios activos (Sutton et al., 2007). La proteína VP6 tiene actividad de unión a ATP y también presenta funciones de helicasa y ATPasa dependiente de RNA. Esta proteína desenrolla la cadena de dsRNA y podría ayudar a la síntesis de mRNA a partir del molde de dsRNA genómico (Stauber et al., 1997). VP6 se asocia con los cuerpos de inclusión formados por la proteína NS2 y desarrolla su actividad desde fases tempranas de la replicación (Matsuo and Roy, 2009).

2.2.4. Proteínas no estructurales.

Las proteínas no estructurales de BTV se producen durante la infección celular y están implicadas en el control de la replicación y la liberación de la progenie de partículas virales (Hyatt, Zhao, and Roy, 1993). Las proteínas NS1 y NS2 son las que más se expresan en células de mamífero infectadas por el virus (Huismans and Van Dijk, 1990) mientras que NS3 y NS3A se sintetizan en mucha mayor cantidad en células de insecto en comparación con la expresión en células de mamífero, con lo que su principal función puede estar relacionada con la

replicación y diseminación de BTV dentro del insecto vector (Guirakhoo, Catalan, and Monath, 1995). En los últimos años se han descubierto dos nuevos marcos de lectura que corresponden con las proteínas no estructurales NS4 y NS5.

La formación característica de estructuras tubulares citoplasmáticas durante la infección por BTV y otros orbivirus es debido a la expresión de la proteína no estructural NS1, la mayor entre las no estructurales (64 KDa) (Lecatsas, 1968; Murphy et al., 1971). NS1 es la proteína más abundante expresada durante la infección. Su expresión se da desde las fases más tempranas de la replicación (Hwang et al., 1993)y supone hasta el 25% de las proteínas del virus presente en las células infectadas, ensamblándose rápidamente en estas estructuras tubulares citoplasmáticas (Huismans, 1979). También se ha observado que pueden asociarse con las factorías virales (Brookes, Hyatt, and Eaton, 1993). Aunque las propiedades funcionales de estos túbulos todavía no son claras, se ha sugerido que pueden estar involucrados en la regulación de la síntesis de las proteínas virales y la patogénesis (Boyce, Celma, and Roy, 2012; Owens, Limn, and Roy, 2004), además de poseer la capacidad de anclarse a los filamentos intermedios del citoesqueleto de la célula (Eaton, Hyatt, and Brookes, 1990) y podrían estar implicados en el proceso de transporte de las partículas virales hacia la membrana de la célula antes de su liberación. Animales infectados por BTV presentan unos altos títulos de anticuerpos dirigidos frente a NS1, debido probablemente a esta abundancia de NS1 en el citoplasma (Richards et al., 1988), por lo que se ha considerado su uso en test de diagnóstico por ELISA (Anderson, Mertens, and Herniman, 1993). Esta proteína se ha usado como un soporte de péptidos exógenos para generar una vacuna heteróloga frente al virus de la inmunodeficiencia adquirida (HIV) (Larke et al., 2005), de la fiebre aftosa y de la gripe (Ghosh, Borca, and Roy, 2002).

La proteína NS2 es el principal constituyente de los cuerpos de inclusión o factorías virales (lugares de replicación y ensamblaje) que se observan en células infectadas, mayoritariamente en la proximidad del núcleo (Brookes, Hyatt, and Eaton, 1993; Thomas, Booth, and Roy, 1990). Se ha descrito que NS2 se une al ssRNA viral por su región N-terminal. Esta proteína podría tener un papel fundamental en la selección de los segmentos de ssRNA para su incorporación a la progenie viral, antes de la encapsidación y replicación del genoma (Fillmore, Lin, and Li, 2002; Lymperopoulos et al., 2006; Lymperopoulos et al., 2003; Zhao et al., 1994). NS2 hidroliza los nucleótidos trifosfato a nucleótidos monofosfato (Horscroft and Roy, 2000). Además, existen evidencias bioquímicas de que la proteína NS2 puede atraer a las proteínas del core (VP1, VP3, VP4, VP6 y VP7), interaccionando con VP7 solo cuando NS2

forma un complejo con VP3, pero no es necesaria su participación en las fases tempranas del ensamblaje (Kar, Bhattacharya, and Roy, 2007).

El segmento 10 de BTV codifica tres proteínas no estructurales; NS3/NS3A y NS5 (Mertens, Brown, and Sangar, 1984; Stewart et al., 2015). El primer marco de lectura abierto (ORF) codifica la proteína NS3 y un segundo punto de inciación de la traducción codifica la proteína NS3A, versión truncada de NS3, la cual carece de 13 aminoácidos de la región N-terminal. Un segundo ORF de este segmento da lugar a la proteína NS5. NS3 y su forma más pequeña NS3A son las únicas proteínas de membrana codificadas por los orbivirus. Parecen estar asociadas con orgánulos implicados en las vías de secreción como el aparato de Golgi, la superficie de vesículas y también en la membrana plasmática durante la salida del virus desde la célula infectada (Hyatt et al., 1991; Wu et al., 1992). Se ha descrito que la proteína NS3 funciona como una viroporina, facilitando la liberación del virus mediante permeabilización de la membrana (Han and Harty, 2004). Por otra parte, también se ha observado que NS3 se ancla a la proteína celular Tsg101 (Wirblich, Bhattacharya, and Roy, 2006) e interacciona con S100A10/p11 (proteína de unión a calcio), permitiendo que las partículas de BTV se liberen de la célula infectada por un mecanismo de "budding", similar al de los retrovirus. Se ha observado que la expresión de NS3/NS3A es mucho más elevada en células de insecto en comparación con células de mamífero, pudiendo jugar un papel esencial en la liberación del virus en las células del vector (Guirakhoo, Catalan, and Monath, 1995; Hyatt, Zhao, and Roy, 1993; Van Dijk and Huismans, 1988), donde no se observa efecto citopático. Otros estudios basados en técnicas de genética reversa han puesto de manifiesto la necesidad de que NS3 esté presente para que el virus se propague eficientemente (virus sin la proteína NS3 están fuertemente atenuados) (Celma and Roy, 2011). En este mismo estudio se hacen deleciones en la proteína NS3, observando que las partículas virales no se liberan eficientemente debido a la falta de interacción de esta proteína con S100A10/p11, indicando que la región N-terminal es esencial en la salida del virus en las células de mamífero. Sin embargo, en las células de insecto es esencial tanto NS3 como NS3A. En la replicación en células de mamífero se ha visto que su participación no es esencial (van Gennip, van de Water, and van Rijn, 2014), mientras que en las células del vector (*Culicoides sonorensis*), virus delecionados de NS3/NS3A no son capaces de replicar ni propagarse (Feenstra et al., 2015). Por último se ha descrito que NS3 actúa como un antagonista de Interferón tipo I en células de mamífero (Chauveau et al., 2013; Ftaich et al., 2015).

Recientemente se ha encontrado un segundo ORF en el segmento 10, correspondiente a la proteína NS5 (Stewart et al., 2015). Es una pequeña proteína de entre 50-59 aminoácidos

que parece localizarse en el nucléolo (Stewart et al., 2015). Virus delecionados para esta proteína no parecen tener alterado su capacidad de replicación y sorprendentemente son altamente patogénicos en el modelo de ratón, sugiriendo que NS5 participa de forma activa en el control de la patogenicidad (Stewart et al., 2015).

En el segmento 9, el cual codifica la proteína VP6, aparece un segundo ORF que codifica la proteína no estructural NS4 de entre 77-79 aminoácidos (Belhouchet et al., 2011; Ratinier et al., 2011). Mediante ensayos de expresión y microscopía confocal se ha observado que esta proteína se detecta tanto en el citoplasma como en el núcleo de células BHK y KC (Belhouchet et al., 2011), además de localizarse en el nucléolo durante las fases tempranas de la infección (Ratinier et al., 2011). Poco se conoce sobre la función de esta proteína, pero estudios preliminares parecen indicar que tiene una función protectora del DNA frente a la degradación por proteasas, no conociéndose con precisión el propósito de esta función. Además, NS4 es un antagonista del interferón tipo I y su expresión confiere al virus una ventaja adaptativa durante la replicación frente al estado antiviral de la célula (Ratinier et al., 2011).

2.3. *Ciclo de replicación de BTV.*

El ciclo de replicación de BTV comienza con la unión del virión a la superficie celular por interacción entre VP2 y los receptores celulares (Eaton and Crameri, 1989; Forzan, Wirblich, and Roy, 2004; Hassan and Roy, 1999). Las partículas del virus se internalizan en endosomas revestidos por una capa exterior de clatrina (Forzan, Marsh, and Roy, 2007). Recientemente, se ha descrito que BTV puede llevar acabo otras vías de endocitosis como la macropinocitosis (Gold et al., 2010) que no requiere de receptores celulares específicos. El aumento de la acidez, debido al bajo pH del interior de los endosomas, provoca que se desensamble la cápsida proteica exterior (Eaton, Hyatt, and Brookes, 1990) y el core transcripcionalmente activo se libera al citosol celular (Huismans, van Dijk, and Els, 1987). La capacidad de permeabilización de los dominios de VP5 da dar lugar a la formación de poros en la membrana endosomal (Hassan and Roy, 1999) por donde se liberara el core, mientras que las proteínas de la cápsida exterior quedan retenidas en el endosoma. Por otra parte, se ha observado que el core es también infectivo por sí mismo ya que VP7 puede a su vez mediar el anclaje y la internalización en la célula, posiblemente a través de un mecanismo distinto (Mertens et al., 1996). Como otros miembros de la familia *Reoviridae*, BTV replica dentro del citoplasma de las células infectadas. Dentro del core, la proteína VP1 transcribe las copias de sentido positivo de ssRNA de cada uno de los diez segmentos genómicos de BTV (Boyce et al., 2004). A los RNA mensajeros resultantes se les añade un residuo “cap” por medio de la

proteína VP4 (Sutton et al., 2007) y se liberan al citoplasma celuar por canales situados en los ejes del core (Mertens and Diprose, 2004). Los mRNA virales sirven como molde para la traducción de las proteínas virales, que tiene lugar aproximadamente 2 horas después del inicio de la infección (Diprose et al., 2001). Los ssRNA positivos son dirigidos a los cuerpos de inclusión viral (VIB) formados por NS2 cuando esta se encuentra fosforilada en al menos uno de sus dos sitios de fosforilación (Modrof, Lymperopoulos, and Roy, 2005) y se encapsidan los diferentes segmentos dentro de la capa formada por la proteína VP3. Este proceso puede implicar interacciones con las proteínas NS2 (Kar, Bhattacharya, and Roy, 2007), VP1, VP4 y la helicasa VP6 (Stauber et al., 1997). Posteriormente, la proteína VP1 sintetiza las cadenas negativas de RNA para producir los RNAs de doble cadena (Boyce et al., 2004). Se ha propuesto que cada segmento de dsRNA se asocia con un complejo de transcripción diferente (VP1, VP4 y VP6) localizado por debajo de la capa formada por VP3, a lo largo de los 5 ejes observándose una forma de flor por criomicroscopía (Nason et al., 2004). Cuando dos serotipos o cepas distintas de BTV infectan una misma célula, se pueden producir intercambios de segmentos de dsRNA, contribuyéndose a la evolución de BTV a través de un proceso de reorganización génica (*reassortant*). Este proceso puede implicar la fusión de cuerpos de inclusión formados con diferentes partículas virales en su interior. Por razones desconocidas, algunos segmentos se intercambian con más frecuencia que otros (Gould and Hyatt, 1994). La capa de VP3 es una estructura relativamente frágil e inestable (Roy, 1992) que sirve como soporte para la adición de trímeros de VP7, dando mayor rigidez y estabilidad al core. Las proteínas VP2 y VP5 parecen ser añadidas a la superficie del core en la periferia de los VIB. La progenie de partículas virales maduras son transportadas dentro del citoplasma a través de microtúbulos debido a la interacción de VP2 con la vimentina (Bhattacharya, Noad, and Roy, 2007). La liberación de los viriones fuera de la célula infectada ocurre por la desestabilización de la membrana celular mediante la actividad viroporina de la proteína NS3 (Han and Harty, 2004), en algunos casos vía “budding”, o como resultado de la lisis o muerte celular. Los 10 segmentos del genoma viral siempre permanecen empaquetados dentro del core a lo largo del ciclo de replicación, lo que ayuda a prevenir la activación de los mecanismos de defensa de la célula infectada que se desencadenarían al darse un contacto directo entre el dsRNA y el citoplasma de la célula. Por tanto para evitar esto la replicación del genoma y la transcripción deben tener lugar dentro del core (Jacobs and Langland, 1996). Se ha observado que conforme la infección progresa, los VIBs se desplazan a lo largo de los microtúbulos hacia regiones perinucleares (Eaton, Hyatt, and Brookes, 1990). La capacidad de la proteína NS2 para unirse al ssRNA indica que probablemente esté implicada en el ensamblaje, replicación y empaquetamiento genómico de BTV (Fillmore, Lin, and Li, 2002; Lymperopoulos et al., 2006; Lymperopoulos et al., 2003). Los

r d

viriones recién formados son transportados a través de la célula y libreados por budding o por desestabilización de la membrana celular, facilitado por la proteína NS3 y probablemente por NS1 (Guirakhoo, Catalan, and Monath, 1995; Hyatt, Zhao, and Roy, 1993; Van Dijk and Huismans, 1988). Poco se conoce acerca de la función de NS1, además de que forma túbulos en el citoplasma y de que se observa su expresión a partir de las 2 horas post-infección. Tras la liberación de la nueva progenie viral, el ciclo de replicación se repite en las células adyacentes (Figura 2).

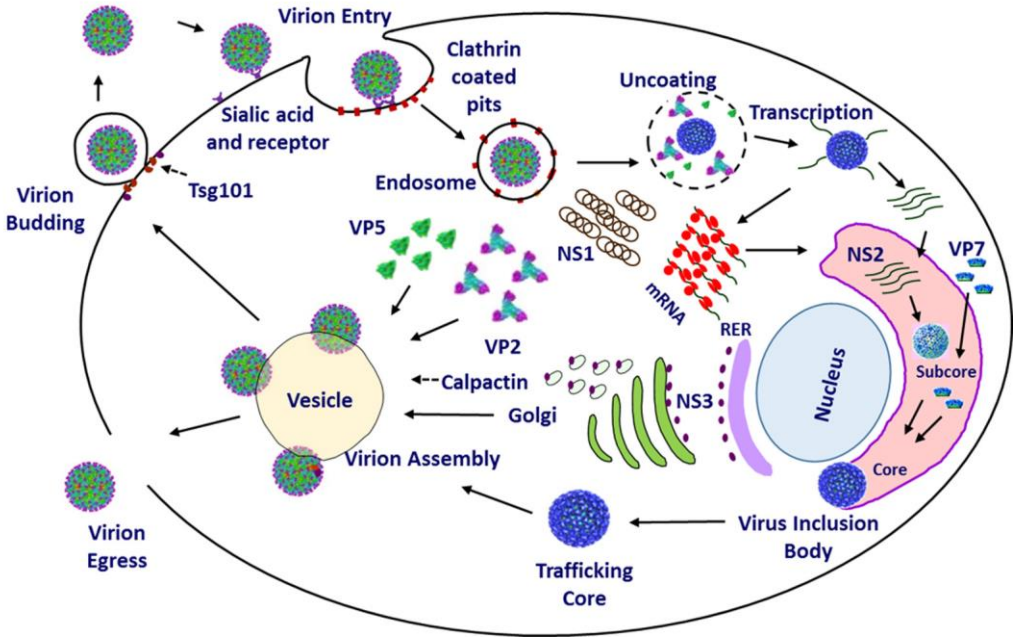


Figura 2. Representación del ciclo de replicación de BTV (Patel and Roy, 2014).

3. El papel del vector en la transmisión y persistencia.

BTV se transmite entre rumiantes principalmente mediante la mordedura de insectos del género *Culicoides* (Orden *Diptera*, Familia *Ceratopogonidae*), si bien no todas las especies de *Culicoides* resultan vectores eficientes del virus (Gibbs and Greiner, 1994; Tabachnick, 2004). Existen aspectos importantes a considerar en cuanto a los vectores competentes, como es la preferencia por el hospedador o las actividades circadiana y estacional (Kampen, 2009). Además, dentro de una especie competente de *Culicoides*, no todas las hembras son

susceptibles a la infección por BTV o capaces de transmitir el virus (Mellor, 1990). El principal vector biológico de BTV en el sur de Europa es *C. imicola*, y fue la primera especie a la que se le reconoció su papel en la transmisión de BTV. Sin embargo, desde 2006 la enfermedad se ha dispersado por el norte de Europa más allá del alcance de esta especie, lo que ha resultado en la implicación de otras especies de *Culicoides* (*C. obsoletus, C. pulicaris*), aunque también se ha visto una ampliación en la distribución geográfica de *C. Imicola* debido a los efectos del cambio climático (Purse et al., 2005). Otros artrópodos pueden propagar el virus por transmisión mecánica (Luedke, Jochim, and Bowne, 1965). Por otra parte se ha documentado que el serotipo 8 de BTV, que ha circulado en Europa desde 2006, es capaz de infectar garrapatas (Bouwknegt et al., 2010). Hasta el momento, estos otros vectores de BTV no se considera que tengan importancia epidemiológica. La distribución geográfica y la incidencia estacional de BTV están determinadas en gran medida por la presencia de los vectores apropiados (Venter et al., 2009). Las distintas especies de *Culicoides* que son responsables de la transmisión de BTV tienen una distribución determinada en diferentes regiones del mundo (Maclachlan et al., 2009). La estacionalidad de BTV está influenciada por factores ecológicos como son las lluvias, la temperatura y la humedad (Purse et al., 2005). Durante su periodo activo, las hembras adultas de *Culicoides* se alimentan de sangre para obtener las proteínas necesarias para la producción de huevos. Si el rumiante al que muerden está infectado con BTV, las partículas virales presentes en su sangre replicarán en las células epiteliales del intestino medio del insecto. Después la progenie viral se libera a la hemolinfa e infecta órganos diana secundarios. La transmisión a otro rumiante por parte del insecto sólo es posible después de que el virus haya replicado en sus glándulas salivares y esto ocurre entre 4 y 20 días después de la infección inicial del insecto, dependiendo de la temperatura ambiental (Purse et al., 2005) (Figura 3). Una vez que el insecto es infectado con BTV, permanece infectado de por vida, sin aparente desventaja para él (Mellor, 1990). Puesto que la hembra de *Culicoides* se alimenta cada 4 días aproximadamente, en poco tiempo puede verse infectado un gran número de animales, a lo que se le añade la aparición de una viremia temprana. Hay una evidencia molecular de transmisión vertical en *Culicoides* (White et al., 2005) pero el virus nunca ha sido aislado a partir de larvas. Existen otras vías de transmisión de BTV distintas a la transmisión por vectores. Por ejemplo, el semen de toros que están en fase virémica puede ser infeccioso y se ha documentado la infección de vacas por este medio (Bowen, Howard, and Pickett, 1985). Por otra parte existen varias evidencias de transmisión horizontal entre rumiantes. La más antigua es la infección experimental de ovejas por administración oral del virus (Jochim, Luedke, and Bowne, 1965). Se ha demostrado que el calostro de vacas virémicas no contiene virus infeccioso (Backx et al., 2009). En cambio, hay varios estudios que documentan la infección de

animales por ingestión de placenta de un animal infectado con lengua azul, en concreto con la cepa europea de BTV-8 (Menzies et al., 2008). También ha habido casos de infección de animales sanos en contacto con animales infectados (Lopez-Olvera et al., 2010). Se ha observado que el recientemente descubierto BTV-26 se transmite por contacto directo, siendo incapaz de hacerlo a través de la mordedura del vector (Batten et al., 2014; Batten et al., 2013). Estudios llevados a cabo en nuestro laboratorio también han confirmado la transmisión oral del virus en el modelo de ratón utilizado para el estudio de este virus, el ratón IFNAR(-/-) (Calvo-Pinilla, Nieto, and Ortego, 2010). La transmisión vertical con potencial teratogénico por parte de las vacunas vivas atenuadas de BTV ha sido descrita años, pero se desconocía para las cepas de campo. El pase de cepas de BTV a través de cultivos celulares puede conferirles la indeseable capacidad de atravesar la placenta, provocando malformaciones fetales y abortos (Schultz and Delay, 1955). Hoy en día, esos efectos están bien reconocidos y el uso de estas vacunas vivas atenuadas en hembras preñadas está totalmente desaconsejado (Osburn, 1994). En cambio, es menos usual que la exposición a cepas silvestres de BTV pueda causar una infección fetal. En regiones donde no se han usado nunca vacunas atenuadas como Australia, no hay evidencia del paso de BTV a través de la placenta (Kirkland and Hawkes, 2004). Sin embargo, se han confirmado infecciones transplacentales en vacas y ovejas con la cepa europea de BTV-8, tanto en estudios experimentales (Backx et al., 2009; Menzies et al., 2008; Wouda et al., 2009) como en diferentes estudios de campo (Darpel et al., 2009; Saegerman et al., 2011; Santman-Berends et al., 2010). También se ha descrito la transmisión transplacental en cabras con el serotipo 25 de BTV denominado Toggenburg (TOV) (Chaignat et al., 2009). La gran capacidad de este virus para intercambiar segmentos génicos entre serotipos y su alta tasa de mutación le han permitido adoptar múltiples estrategias de transmisión.

En cuanto a la persistencia del virus de un año para otro, se ha descrito que las especies de *Culicoides* propias del hemisferio norte sobreviven al invierno en su estado larvario, considerando éste el mecanismo más probable de persistencia interanual (*overwintering*), siendo el virus transmitido transováricamente desde el vector infectado a los huevos. Un estudio puso de manifiesto la presencia de RNA viral en las larvas de *Culicoides*, aunque no se pudo rescatar virus viable (White et al., 2005). Se ha visto que el resto de vectores que no pertencen al hemisferio norte, aún siendo menos tolerantes que los vectores del género Culex a las bajas temperaturas, son capaces de sobrevivir a los inviernos suaves (Mellor, Boorman, and Baylis, 2000). Estudios llevados a cabo en laboratorio han recreado estas condiciones ambientales, observándo que la esperanza de vida de los *Culicoides* puede extenderse de 10-20 días hasta tres meses si la temperature se mantiene a 10ºC (Lysyk and Danyk, 2007). Estudios que apoyan este potencial mecanismo de persistencia se han basado

en la captura de *Culicoides* durante el invierno suave de 2006-2007 en Europa y su utilización para infectar mamíferos al año siguiente (Zimmer et al., 2008). Sin embargo, no queda claro que estos individuos que sobreviven a los inviernos templados puedan mantener una actividad continua a la hora de alimentarse de la sangre de los hospedadores, debido a posibles cambios en su comportamiento, como puede ser la búsqueda de los hospedadores cuando éstos están estabulados (Wilson, Darpel, and Mellor, 2008). También se ha estudiado el potencial de persistencia del virus en el rumiante. Tradicionalmente se había pensado que tras 60 días post-infección no era possible la recuperación de virus infeccioso a partir de sangre de rumiantes infectados (Barratt-Boyes and MacLachlan, 1994; Heidner et al., 1988; Luedke, 1969; Luedke, Jochim, and Jones, 1969; MacLachlan et al., 1992; Richards et al., 1988), pero paradójicamente, se han detectado muestras positivas por PCR tras más de 200 días post-infección (MacLachlan et al., 1994). Otros trabajos muestran que es possible el aislamiento de virus infeccioso a partir de piel de una oveja infectada a las 6 semanas post-infección, en ausencia de virus infeccioso en sangre (Takamatsu et al., 2003). Se ha descrito que las células T γδ ovinas pueden permanecer persistentemente infectadas con BTV en ausencia de efectos citopáticos visibles cuando se cultivan *ex vivo* (Stott et al., 1990; Takamatsu et al., 2003) lo que apoya la propuesta de que el virus pueda permanecer de forma latente en el hospedador de un año para otro. Por el contrario, en otros studios similares no se ha conseguido reproducir estos resutados (Lunt et al., 2006; Melville et al., 2004; White and Mecham, 2004). Aún con toda esta información, todavía no queda claro cómo el virus puede sobrevivir durante largos periodos de tiempo en los vectores adultos y resurgir en las épocas más favorables, por lo que el principal mecanismo de persistencia todavía no ha sido confirmado.

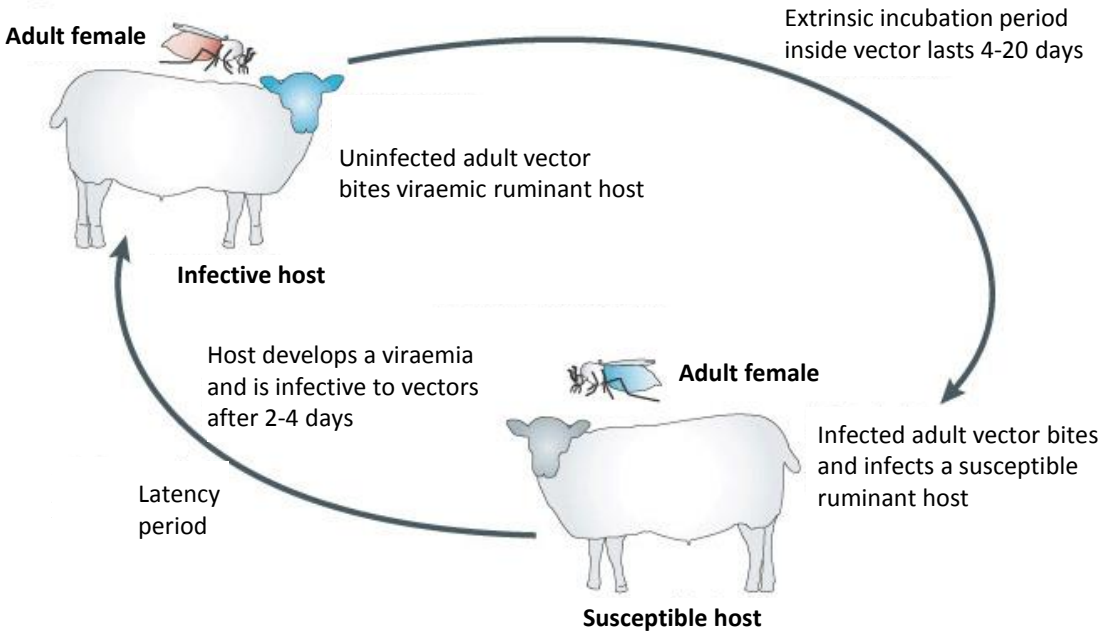


Figura 3. Ciclo de transmisión del virus entre rumiantes mediante *Culicoides (Purse et al., 2005).*

4. *Hospedadores y células diana.*

BTV infecta principalmente a rumiantes de todas las especies tanto silvestres como domésticos siendo el rango de hospedadores muy amplio: ovejas, vacas, cabras, ciervos, muflones y antílopes entre otros. En menor medida, también es común la infección de BTV en camélidos como las alpacas. Probablemente los primeros hospedadores vertebrados fueron los antílopes africanos. Aunque los rumiantes son los hospedadores más importantes de BTV, este virus parece que puede infectar otras especies. En los años 90, una vacuna canina contaminada con BTV-11 provocó abortos y muertes en perros que habían sido infectados accidentalmente con dicha vacuna (Wilbur et al., 1994). Esto fue corroborado en experimentos posteriores (Brown et al., 1996). Por otra parte, existen evidencias de una infección natural de BTV en carnívoros africanos, posiblemente como resultado de la ingestión de rumiantes infectados con lengua azul, ya que los altos niveles de anticuerpos frente a BTV sugieren que ha habido cierto nivel de replicación del virus en los carnívoros (Alexander et al., 1994). También se documentó la muerte de dos linces euroasiáticos después de ser alimentados con fetos de rumiantes infectados con BTV-8 y el estudio postmortem reveló que los linces eran seropositivos para BTV y además, uno de ellos por PCR (Jauniaux et al., 2008). Otro caso relacionado es la infección con AHSV de carnívoros que fueron alimentados con carne de caballo infectado (Van Rensberg et al., 1981). En cuanto a animales de laboratorio, durante

más de 50 años se ha utilizado la infección intracraneal de ratones neonatos para la investigación y producción de vacunas (Franchi et al., 2008; Svehag, 1962; Van Den Ende, Linder, and Kaschula, 1954). Además, se ha observado cómo ratones transgénicos delecionados en la subunidad β del gen del receptor de IFNα/β son susceptibles a la infección, desarrollando una patología similar a la que sufren los hospedadores naturales infectados (Calvo-Pinilla et al., 2009a). La infeccion en el rumiante se caracteriza por una patología hemorragica aguda, lo que sugiere que el virus tiene tropismo por las células endoteliales, comfirmado por técnicas de PCR, hibridación *in situ* e inmunofluorescencia (Brodie et al., 1998). Además, se ha observado efecto citopatico en cultivos primarios de células endoteliales tanto ovinas como bovinas, derivadas de la arteria pulmonar y de capilares pulmonares (Barratt-Boyes et al., 1992; DeMaula et al., 2001; Takamatsu and Jeggo, 1989). La replicación del virus en celulas endoteliales se ha confirmado mediante microscopía confocal, usando anticuerpos dirigidos tanto a las proteínas estructurales como a las no estructurales, presentes solo durante la infección (Darpel et al., 2012). Subpoblaciones específicas de leucocitos también son susceptibles a la infección por BTV (Barratt-Boyes et al., 1992) como linfocitos, macrofagos, neutrofilos, células dendriticas y monocitos (Barratt-Boyes et al., 1992; Maclachlan et al., 2009; Stott et al., 1990; Takamatsu and Jeggo, 1989). La interacción de BTV con eritrocitos y linfocitos no estimulados no va más allá del anclaje del virus a su membrana celular (Brewer and MacLachlan, 1994).

En cuanto a las células diana de BTV en el vector artrópodo se ha observado que BTV replica en las células intestinales tras la ingestion de sangre procedente del ruminate infectado. Los virus pasan al hemocele y a los adipocitos antes de replicar en las glándulas salivares. Solo los individuos donde el virus ha replicado en las glándulas salivares son capaces de transmitir el virus a otros hospedadores susceptibles (Mellor, 2009).

5. *Enfermedad y signos clínicos.*

Las manifestaciones clínicas de la enfermedad de la lengua azul varían considerablemente entre las distintas especies de hospedadores y las cepas virales (Maclachlan et al., 2009). El serotipo no determina la virulencia ya que existen cepas altamente virulentas y otras más benignas dentro del mismo serotipo (Kirkland and Hawkes, 2004; Maclachlan et al., 2009). Aunque BTV afecta a muchas especies de rumiantes, el cuadro clínico completo de la enfermedad aparece generalmente en la oveja (Erasmus, 1990). Los signos clínicos pueden ser fiebre alta durante algunos días así como congestión, descarga

ocular y nasal, apatía, rigidez muscular y cojera (Wilson, Darpel, and Mellor, 2008). Se da un deterioro de la lana y del estado general del animal, además se produce una pérdida de peso y se reduce la producción de leche, causando graves pérdidas económicas en la producción. Las lesiones típicas son edema facial, inflamación del rodete coronario (coronitis), laminitis, necrosis en músculo esquelético y cardíaco (MacLachlan et al., 2008), edemas y úlceras necróticas en la mucosa oral, edema pulmonar y trombosis vascular (MacLachlan et al., 1994). Algunas veces se produce cianosis de la lengua, lo que da nombre a la enfermedad de la lengua azul, pero es un signo clínico poco común. Otro signo clínico de la enfermedad es la aparición de hemorragias en la base de la arteria pulmonar (Roy, 2007). Como excepción, las razas de ovejas de las regiones donde BTV es endémico, raramente se ven afectadas; en cambio hay otras razas de ovejas que son particularmente susceptibles (Jeggo et al., 1987; Veronesi, Hamblin, and Mellor, 2005; Worwa et al., 2010). Por otra parte, hay factores como la exposición a la luz solar (Erasmus, 1990) o como la inmunodeficiencia provocada por infecciones simultáneas (Brodie et al., 1998) que pueden exacerbar la gravedad de la enfermedad. En general, la morbilidad es variable y puede llegar a ser del 100%. Por lo que respecta a la tasa de mortalidad, varía mucho entre especies y serotipos. Una infection aguda severa puede llevar a la muerte del animal en unos 14 días. El resto de rumiantes a los que infecta el virus no suelen desarrollar signos clínicos, sobre todo en el caso de animales silvestres. El ganado bovino es considerado como un reservorio importante debido a que desarrolla una viremia prolongada y por lo general, la enfermedad es subclínica. Pero aunque este ganado no suele desarrollar signos clínicos, el brote europeo de BTV-8 causó la enfermedad a un gran número de bovinos como nunca antes se había visto, con una gran incidencia de edemas, coronitis, disnea, conjuntivitis lesiones en las mamas y en la mucosa nasal (Elbers et al., 2008). Aun así, también en esta epidemia, la tasa de mortalidad fue mayor en ganado ovino que en bovino (Conraths et al., 2009). En ganado caprino, la enfermedad de la lengua azul suele ser leve o inaparente (Backx et al., 2007). La patología de BTV está asociada a un daño en el endotelio vascular, resultado de cambios en la permeabilidad y fragilidad de los capilares, con la consiguiente diseminación de la coagulación intravascular y necrosis de los tejidos a los que riegan esos capilares dañados (Mahrt and Osburn, 1986). Se ha visto que la infección de BTV activa células endoteliales pulmonares y macrófagos (DeMaula et al., 2001; DeMaula et al., 2002b; Drew et al., 2010b). Existe una similitud con otras fiebres hemorrágicas virales como es el Ébola, donde las citoquinas proinflamatorias producidas por el hospedador (como IL-1β) y otras sustancias vasoactivas (óxido nítrico) contribuyen a la disfunción endotelial y al incremento de la permeabilidad vascular, mediado principalmente por el factor TNF, liberado por macrófagos y otras células locales del sistema immune. Esto conlleva a que

en algunos casos la enfermedad de la lengua azul sea fulminante (DeMaula et al., 2002a; DeMaula et al., 2002b; Drew et al., 2010b; Howerth, 2015; Maclachlan et al., 2009).

Tras la infección por el vector, BTV comienza a replica en las células diana de la piel y es transportado a los nódulos linfáticos regionales por células dendríticas migradoras (Darpel et al., 2012; Hemati et al., 2009). Después de esta replicación primaria, se produce una replicación secundaria en otros nódulos linfáticos, timo y bazo (Barratt-Boyes et al., 1995; Pini, 1976). La progenie viral producida es liberada al torrente sanguíneo donde se producen grandes cantidades de virus en las células sanguíneas mononucleares periféricas (PBMC) y en el endotelio infectados, lo que conduce a la necrosis vascular y al daño tisular (Barratt-Boyes and MacLachlan, 1994; Drew et al., 2010a; Mahrt and Osburn, 1986; Pini, 1976). En rumiantes la viremia puede persistir durante periodos largos de tiempo, incluso en presencia de anticuerpos neutralizantes (Luedke, 1969; Luedke, Jochim, and Jones, 1969; Singer, MacLachlan, and Carpenter, 2001). Se puede recuperar virus infeccioso de la sangre de los rumiantes infectados durante aproximadamente dos meses, mientras que el RNA viral puede ser detectado durante más tiempo (Katz et al., 1994). Esto es debido a la asociación pasiva de BTV con los eritrocitos (Brodie et al., 1998; MacLachlan et al., 1994) y el periodo de vida de los mismos se corresponde con el tiempo durante el que se detecta RNA de BTV en sangre (Brewer and MacLachlan, 1994). Los viriones de BTV se unen a glicoforinas en la superficie de los eritrocitos de los rumiantes y escapan de la neutralización en invaginaciones de su membrana, alargándose así el tiempo durante el cual nuevos vectores pueden transmitir la infección (Eaton and Crameri, 1989). Aunque la viremia es prolongada, sobre todo en ganado bovino, no es persistente, y los animales que se recuperan serán inmunes a una reinfección por el mismo serotipo (MacLachlan et al., 1990).

6. *Epidemiología, distribución e impacto global de la lengua azul.*

La legua azul fue reconocida por primera vez cuando se introdujeron ovejas merinas en Sudáfrica a finales del siglo XVIII (Spreull, 1905; Verwoerd, 2004), aunque seguramente la infección era endémica en rumiantes africanos desde la antigüedad. Spreull (1905) concedió a Robertson y Theiler el descubrimiento de la naturaleza vírica de la enfermedad y sugirió el nombre de lengua azul, que ya estaba siendo utilizado por los granjeros africanos ("*bluotong*") debido a la cianosis que se observaba en la lengua de las ovejas más graves. En 1948 fue la primera vez que se describió la pluralidad de las diferentes cepas de BTV, su virulencia variable y las implicaciones de la inmunización frente a BTV (Neitz, 1948). Más tarde, se clasificó el

serogrupo de BTV en diferentes serotipos, que se diferenciaron mediante ensayos de seroneutralización, basados en la pérdida de reacción cruzada en la neutralización. En un principio se definieron 16 serotipos (Howell, 1960), pero este número fue incrementándose consecutivamente hasta 24 (Gorman, 1990). Los aislados de un mismo serotipo con distinto origen geográfico (topotipos) se pueden diferenciar por las variaciones de secuencia en el segmento genómico 2 (Mertens et al., 2007), aunque también presentan variaciones en otros segmentos genómicos. La separación geográfica permite la adquisición de puntos de mutación únicos (Maan, 2009a). Estas variaciones se pueden usar para dividir las distintas cepas de BTV dentro de un grupo de aislados del Este (Medio y lejano oriente, incluyendo Australia e India), y un grupo de aislados del Oeste (incluyendo África y América) (Balasuriya, Nadler et al. 2008). En Europa han ocurrido brotes causados por cepas tanto del Este como del Oeste.

Hasta los años 40, la lengua azul fue una enfermedad endémica y exclusiva del continente africano, pero a partir de entonces se ha extendió por todos los continentes menos la Antártida. El primer brote de BTV confirmado fuera de África fue en Chipre en 1943 (Gambles 1949), y estuvo seguido de otros en Israel (Komarov 1951), Pakistán (Sarwar 1962) y en India (Sapre 1964). En los años en 50, se confirmó la infección de BTV en ovejas en California (Hardy and Price, 1952; McKercher et al., 1953). Sin embargo los análisis genéticos posteriores indicaron que algunos serotipos de BTV comparten una larga historia evolutiva en América del Norte (Heidner et al., 1991). Actualmente la distribución geográfica de este virus es muy amplia. La enfermedad se ha descrito históricamente en un área geográfica entre el paralelo 35º sur y el paralelo 42º norte en África, Europa, Asia y Australia, y entre el paralelo 30º sur y el 50º norte en América, con variaciones locales en relación a las condiciones climáticas y ambientales. Sin embargo, en el año 2006, con la aparición de brotes de BTV-8 en el norte de Europa, el virus cruzó el límite norte que se había descrito previamente (Toussaint et al., 2006). Hasta entonces, sólo cinco serotipos distintos de BTV (1, 2, 4, 9 y 16) se habían distribuido por amplias zonas de la Europa mediterránea. En España se declaró el primer foco en 1956, causado por BTV-10 (Manso-Ribeiro, 1957). Se llegó a erradicar el virus tras una campaña masiva de cuarentena, sacrificio y vacunación (Gorman, 1990). Más tarde se extendió la enfermedad a otros países europeos como Portugal (1959), Suecia (1960) y Grecia (1989). A partir del año 2000 se declararon diversos focos en España producidos por BTV-2 y BTV-4. En el año 2006 sólo BTV-4 apareció en la península. En 2007, BTV-1 comenzó a afectar a España desde Cádiz hasta el País Vasco y el número de brotes declarados de serotipos 1 y 4 ascendió a 1.276. Mientras que los movimientos de BTV-2, 4, 9 y 16 parecen estar restringidos a la cuenca mediterránea, el área afectada por serotipo 1 se ha extendido recientemente a través de la

Península Ibérica y Francia (Maclachlan, 2010). BTV puede ser introducido en áreas nuevas por el movimiento de rumiantes infectados o por la dispersión de insectos infectados con el ganado o con el viento (Mintiens et al., 2008). Los vientos predominantes pueden transportar *Culicoides* a largas distancias (Ducheyne et al., 2007) y esa es la causa de la rápida expansión de las epidemias de BTV desde el norte de África hasta la Península Ibérica y otros sitios de la Europa mediterránea (Wilson and Mellor, 2008). En agosto de 2006, BTV-8 fue detectado por primera vez en los Países Bajos (Enserink 2006). A finales de 2006, aparecieron nuevos casos en Bélgica, Francia, Luxemburgo y Alemania (Saegerman, Berkvens, and Mellor, 2008). Los brotes cesaron en enero de 2007, cuando la estación de los vectores finalizó pero sorprendentemente el virus reapareció poco tiempo después (Hoffmann et al., 2008). Esta rápida re-emergencia de BTV-8 se debió a la supervivencia del virus durante el invierno. Tras el invierno de 2007, BTV-8 se siguió extendiendo dramáticamente por Europa y finalmente llegó al norte de España a principios del 2008. Un nuevo orbivirus apareció en el 2008 en Suiza y fue detectado en cabras (Hofmann et al., 2008). Se denominó "Toggenburg orbivirus" (TOV) el cual más tarde se idntificó como el serotipo BTV-25 (Chaignat et al., 2009). Por otra parte, los serotipos 6 y 11 aparecieron en el norte de Europa a mediados del 2008; el análisis molecular de estas cepas reveló su similitud con los virus de las vacunas vivas atenuadas que se usan en Sudáfrica y se ha sugerido que derivan en parte de estos virus (De Clercq et al., 2009; Eschbaumer et al., 2010; Vandenbussche et al., 2009), aunque el mecanismo de introducción sigue siendo un misterio. El incremento del comercio global probablemente haya aumentado la frecuencia de introducción de virus exóticos en nuevos ecosistemas y el efecto del cambio climático ha contribuido a que nuevas cepas persistan y se expandan (Wilson and Mellor, 2008). Las consecuencias de esto han sido los cambios en la distribución de BTV en Europa así como en el resto del mundo. Por ejemplo, la detección de 8 nuevos serotipos de BTV en Estados Unidos (Johnson et al., 2006), o la detección de BTV-13 y del vector *C. sonorensis* en Ontario, Canadá (Jewiss-Gaines, Barelli, and Hunter, 2017; Mayo et al., 2017). El virus de la lengua azul ha causado muchas pérdidas económicas en todo el mundo, particularmente en poblaciones más susceptibles de áreas no endémicas. Se estima que el número de ovejas que han muerto o han sido sacrificadas debido a las distintas incursiones de BTV por la cuenca mediterránea desde 1998 a 2005, fue más de un millón (Purse et al., 2005). Por otra parte la epidemia en 2006 de BTV-8 en Europa originó el mayor daño económico causado por un solo serotipo hasta el momento (Wilson and Mellor, 2008). Se consiguió controlar gracias a las campañas de vacunación durante los años 2008-2009, pero posteriormente volvió a resurgir en el verano de 2015. En 2014 una nueva cepa de BTV-4 con segmentos de otros serotipos apareció en el sudeste de Europa, procedente probablemente del norte de Africa,

extendiéndose por la región central del continente durante 2015. A finales de verano de 2016 esta misma cepa se detectó en Montenegro, Bosnia-Herzegovina e Italia. Restringir el movimiento de animales es una medida útil para evitar que el virus se extienda rápidamente. Otros métodos de control que ayudan son la esterilización de machos por irradiación y el uso de insecticidas para controlar los vectores (Verwoerd, 2004). En la figura 4 se muestran las zonas de restricción en el movimiento de ganado en la Unión Europa, debido a la presencia de casos de BTV actuales o de menos de dos años de antigüedad.

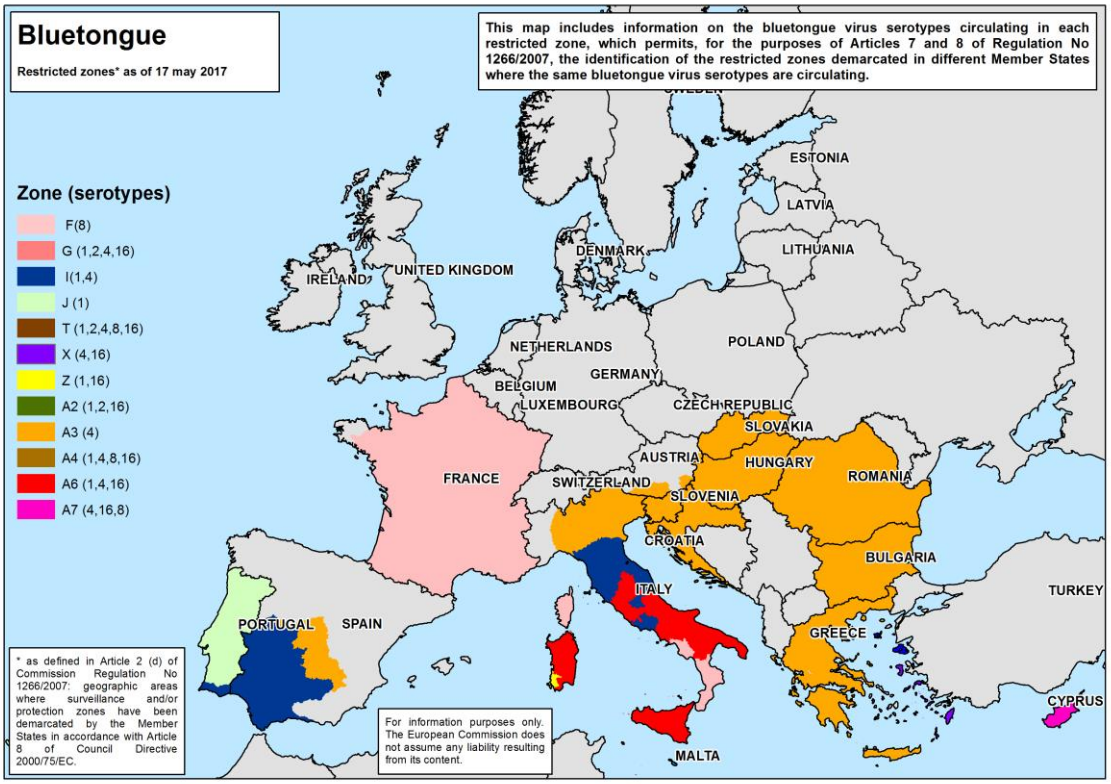


Figura 4. Mapa de zonas en la UE con restricciones de movimiento de ganado a causa de la presencia de BTV (Comisión europea, mayo 2017). La restricción sólo se levanta cuando no hay circulación de BTV en un área durante dos años. https://ec.europa.eu/food/animals/animal-diseases/control-measures/bluetongue_en

7. *Respuesta inmune frente a BTV.*

La infección por BTV causa leucopenia sobre los dias 7 u 8 post-infección (Ellis et al., 1990). Además, BTV es un potente inductor de IFN de tipo I en ovino (Foster et al., 1991), bovino (MacLachlan and Thompson, 1985) y ratón (Jameson, Schoenherr, and Grossberg, 1978). En infecciones naturales se produce una respuesta de anticuerpos neutralizantes que aparece aproximadamente entre 7 y 14 días post-infección (Foster et al., 1991). Estos anticuerpos neutralizantes se dirigen mayoritariamente frente a VP2. Además se ha

determinado que la presencia de la proteína VP5 aumenta la inducción de los anticuerpos neutralizantes a través de su interacción conformacional con VP2 (DeMaula, Bonneau, and MacLachlan, 2000). Los anticuerpos neutralizantes del suero de un animal que se ha recuperado de una infección, pueden proteger pasivamente a otro animal frente a una infección homóloga de BTV, así como los anticuerpos del calostro de una madre infectada naturalmente o incluso vacunada con una vacuna inactivada (Jeggo, Wardley, and Taylor, 1984a; Oura et al., 2010). Pero la infección con un único serotipo no ofrece una protección cruzada duradera frente a otros serotipos ya que sólo se producen anticuerpos neutralizantes frente al serotipo implicado en la infección (Jeggo et al., 1986). En cambio, la infección secuencial de un animal con diferentes serotipos de BTV induce una respuesta amplia de anticuerpos neutralizantes, incluso frente a otros serotipos distintos a los de la infección (Jeggo, Gumm, and Taylor, 1983; Jeggo et al., 1986). Después de la vacunación con las proteínas de la cápsida exterior VP2 y VP5, existe una buena correlación entre la respuesta de anticuerpos neutralizantes y la protección específica frente a un serotipo (Huismans et al., 1987; Roy et al., 1990). Por otra parte, las vacunas de virus completo inactivado pueden proteger frente al desafío de BTV sin llegar a inducir una respuesta apreciable de anticuerpos neutralizantes (Stott, Barber, and Osburn, 1985). De manera similar, el grado de inmunidad inducida después de la infección con BTV no se correlaciona con el nivel de anticuerpos neutralizantes (Jeggo, Wardley, and Taylor, 1984b) sugiriendo la implicación de otros factores en el resultado de la protección frente a la infección. Se ha demostrado la inducción de linfocitos T citotóxicos (CTL, CD8+) específicos frente a BTV en ovejas, vacas (Ellis et al., 1990; Jeggo, Wardley, and Brownlie, 1984) y ratones (Jeggo and Wardley, 1982). En experimentos de transferencia, las ovejas receptoras consiguieron una protección parcial por la transferencia de linfocitos T procedentes de animales gemelos infectados con BTV, no siendo del todo específica de serotipo (Jeggo, Wardley, and Taylor, 1984a; Jochim, 1985). Por otra parte, un experimento de vacunación con la proteína VP7, expresada por un vector viral heterólogo, indujo un cierto grado de protección en ausencia completa de anticuerpos neutralizantes (Wade-Evans et al., 1996). En varios ensayos *in vitro* se determinó que la mayoría de los CTLs específicos de BTV poseen protección cruzada frente a otros serotipos (Jeggo and Wardley, 1985; Takamatsu and Jeggo, 1989). Sin embargo, esta reactividad cruzada parece no correlacionarse con las relaciones entre serotipos en la protección cruzada que generan los anticuerpos neutralizantes, lo que indicaría que en las respuestas inmunes celular y humoral están implicadas diferentes proteínas del virus (Takamatsu and Jeggo 1989). Se conoce poco sobre la localización de epítopos T en las proteínas de BTV. Existen variaciones en cuanto a las proteínas diana de los CTL entre individuos y especies (Schwartz-Cornill, Mertens et al. 2008).

Se ha descrito que en ratones, los CTL reconocen predominantemente las proteínas no estructurales de BTV (Jones, Chuma et al. 1996), mientras que en ovejas se determinó que las proteínas más inmunogénicas para los CTL son VP2, VP7 y NS1 (Andrew et al., 1995; Janardhana et al., 1999; Rojas et al., 2014; Rojas et al., 2011; Takamatsu et al., 1990), encontrando una respuesta más heterotípica cuando está dirigida a las proteínas no estructurales conservadas.

8. *Vacunas frente a BTV.*

8.1. Vacunas atenuadas e inactivadas frente a BTV.

El primer intento de inmunización frente a BTV que se llevó acabo fue poco tiempo después de conocerse la naturaleza viral del agente etiológico que producía la lengua azul en Sudáfrica (Theiler, 1908), utilizándose inóculo de sangre con una cepa poco virulenta de BTV. Esta vacuna se usó durante 4 décadas a pesar de que no era del todo segura y de que no inducía una inmunidad adecuada (Kirkland and Hawkes, 2004). Con el tiempo se seleccionaron cepas que habían sido atenuadas por repetidos pases en embriones de pollo (Alexander, Haig, and Adelaar, 1947). Cuando se optimizaron los sistemas de cultivos celulares para BTV, se empezaron a usar para la propagación de virus, ya que este proceso ofrece la ventaja adicional de poder refinar la cepa vacunal por purificación en placa (Verwoerd, 2004). Existe un balance delicado entre conseguir una reducción adecuada de la virulencia y al mismo tiempo mantener el nivel requerido de eficacia. Se ha visto que las vacunas vivas causan enfermedad en algunas razas de oveja y una viremia corta (Monaco et al., 2006; Veronesi et al., 2010; Veronesi, Hamblin, and Mellor, 2005), pero suficiente para darse la posibilidad de que puedan ser transmitidas por vectores (Ferrari et al., 2005), como ha ocurrido en Italia y Croacia, donde han circulado dos cepas vacunales en poblaciones de animales no vacunados (Ferrari et al., 2005; Listes et al., 2009). Además las cepas atenuadas tienen un tropismo tisular alterado que puede conducir a efectos teratogénicos en animales gestantes (Kirkland and Hawkes, 2004) y el virus puede excretarse en el semen (Kirkland et al., 2004). La ventaja de las vacunas vivas es que la replicación del virus atenuado induce una fuerte respuesta inmune. Aun así son más las desventajas de estas vacunas que las ventajas y actualmente se desaconseja su uso, sobre todo en situación epizoótica.

Los virus con genomas segmentados pueden cambiar su información genética durante una infección simultánea de 2 o más cepas parentales en la misma célula. Por consiguiente, la vacunación con varias cepas de BTV atenuadas, pero capaces de replicar, siempre conlleva el

riesgo de que se produzca una reorganización de segmentos del genoma. El riesgo aumenta cuando las cepas vacunales se transmiten por vectores. Hay muchos ejemplos documentados de reorganización genética en BTV, dentro de un mismo serotipo o entre distintos serotipos (de Mattos et al., 1991; Oberst et al., 1987; Samal et al., 1987a; Samal et al., 1987b; Stott et al., 1987). Por ejemplo se ha visto que el virus BTV-16 aislado en Italia en el año 2002 portaba el segmento 5 de la cepa vacunal de BTV-2. Los primeros experimentos con virus inactivado como vacuna frente a BTV datan de 1975 (Parker, Herniman et al. 1975. Las vacunas inactivadas ofrecen ventajas significativas frente a las vacunas atenuadas, ya que la ausencia de virus replicativo elimina los problemas de viremia, transmisión por vectores, reversión a la virulencia, infección fetal y no hay peligro de que se produzca intercambio de material genético, aunque su coste es mayor y se necesitan adyuvantes en la formulación de la vacuna para aumentar su eficacia (Ramakrishnan et al., 2006). Además, es necesario un control de calidad riguroso para asegurar que cada lote de vacunas está adecuadamente inactivado (Stewart et al., 2010). Se obtuvieron buenos resultados experimentales con vacunas inactivadas monovalentes dirigidas frente a los serotipos 2, 4, 11 y 16 (Hamers et al., 2009; Savini et al., 2008; Stevens et al., 1985), y con la vacuna bivalente dirigida frente a los serotipos 2 y 4 (Savini et al., 2009). Veinticinco años después de los primeros estudios, se usó la primera vacuna inactivada en campo, en concreto en el año 2005, para combatir BTV-2 en Córcega (Savini et al., 2008). Después le siguieron campañas de vacunación con la vacuna inactivada monovalente frente a BTV-4 (en Portugal y España) y con la vacuna inactivada bivalente BTV-2/4 (en Córcega e Italia). Debido al gran impacto de BTV-8 en Europa, se empezó una campaña de vacunación a gran escala en 2008 empleando una vacuna inactivada monovalente frente a ese serotipo, reduciendo significativamente los casos de animales infectados (Gethmann et al., 2009). Por último, también han salido al mercado vacunas inactivadas frente a BTV-1. Una de las ventajas de las vacunas inactivadas es su menor coste de producción. Por otra parte, dependiendo de la pureza de la vacuna inactivada, pueden inducir escasa o nula respuesta inmune frente a las proteínas no estructurales de BTV (Alpar, 2009). A pesar de algunos resultados experimentales prometedores (Barros et al., 2009), no existe un sistema comercial de diagnóstico DIVA para BTV. La mayoría de los sistemas de ELISA disponibles para BTV detectan anticuerpos frente a la proteína VP7, los cuales son inducidos tanto en una infección de BTV como en la inmunización con una vacuna inactivada.

8.2. Antecedentes de vacunas recombinantes frente a BTV.

La ingeniería genética y tecnología del DNA recombinante han facilitado el desarrollo de vacunas recombinantes seguras, pero estas vacunas aún no están disponibles

comercialmente. Esta tecnología ofrece ventajas sustanciales en términos de seguridad y eficacia, y poseen gran potencial para desarrollar vacunas marcadoras. Estas vacunas podrían ser usadas como medida profiláctica en áreas de circulación del virus, sin poner en peligro las áreas libres de BTV de la región. Además, un análisis serológico permitiría distinguir entre animales vacunados e infectados naturalmente (estrategia DIVA). La tecnología de DNA recombinante implica la síntesis de proteínas inmunogénicas que inducen respuestas inmunes protectoras. Varias vacunas recombinantes experimentales han sido descritas teniendo numerosas ventajas potenciales, que incluyen la inducción rápida de inmunidad, la carencia de transmisibilidad y la posibilidad de permitir una estrategia polivalente. Hoy en día, las vacunas recombinantes ya ofrecen tan buena inmunogenicidad como las vacunas basadas en virus atenuados vivos, evitando la posibilidad de que se produzcan reorganizaciones génicas o dispersión del virus por insectos. Hay numerosas posibilidades para generar vacunas frente a BTV que no impliquen virus replicativo. Una opción segura es la vacunación con viriones incompletos o proteínas virales individuales, englobadas en el tipo de vacunas subunidad. La vacunación con la proteína de la cápsida exterior VP2 puede proteger a ovejas frente al desafío con el mismo serotipo de BTV (Huismans et al., 1987). Sin embargo, el título de anticuerpos neutralizantes así como el nivel de protección observada es mayor cuando se usan VP2 y VP5 en la misma inmunización, lo que se debe a la dependencia conformacional entre ellas y a la presencia de epítopos neutralizantes también en VP5, además de en VP2 (DeMaula, Bonneau, and MacLachlan, 2000). Los baculovirus recombinantes han sido usados desde hace tiempo para expresar proteínas de BTV (French, Inumaru, and Roy, 1989). Con las proteínas purificadas se pueden generar estructuras que simulan la partícula viral (VLP), pero carentes de genoma. Estas partículas presentan proteínas estructurales en la conformación correcta generando una buena respuesta inmune (Roy et al., 1994). Las principales desventajas son la baja eficiencia de producción así como su inestabilidad, lo que conlleva un elevado coste de producción y además, en muchos casos, es necesario el uso de adyuvantes para aumentan su inmunogenicidad. Una manera de reducir estos costes se basa en la utilización de plataformas de producción más baratas como las plantas (van Zyl, Meyers, and Rybicki, 2017). Otras estrategias alternativas para la mejora en la inmunogenicidad de las vacunas subunidad se han centrado en la fusión de proteínas de BTV recombinantes a molélulas que facilitan su reconocimiento por parte de las células presentadoras de antígeno y otras células del sistema inmune (Legisa et al., 2015) o la utilización de material particulado que incorpore los antígenos de interés, también mas fácil de reconocer por las células del sistema inmune (Allsopp et al., 1996; Demento et al., 2011; Plebanski et al., 1998).

El uso de vectores DNA para exprersar antígenos también ha sido aplicado en el campo de las vacunas, y actualmente se esta trabajando en su mejora y optimización (Suschak, Williams, and Schmaljohn, 2017). Está descrito que este tipo de vacunas confieren mayoritariamente una respuesta de tipo celular o Th1 y son altamente estables. En el caso de BTV, siempre se han utilizado en combinación con otras estrategias vacunales (Calvo-Pinilla et al., 2012; Jabbar et al., 2013; Li et al., 2015).

La genética reversa ha abierto las puertas para el desarrollo de vacunas vivas. Dentro de este tipo de vacunas están las denominadas "DISA" (*Disabled Infectious Single Animal*) en las que se ha mutado el segmento 10 codificante de la proteína NS3 del genoma de BTV. Así se ha obtenido un virus atenuado con capacidad limitada para propagarse (retrasa la salida del virus, tanto en células de mamífero como en células del vector) pero no para replicar, puesto que su ausencia no afecta a la maquinaria de replicación aunque sí es necesaria para la liberación del virus en las células infectadas (Feenstra et al., 2015), además de actuar como antagonista del IFN-α (Chauveau et al., 2013). Se ha comprobado la eficacia de esta vacuna en ovejas con una sola dosis de $2x10^5$ TCID de distintos serotipos de BTV (1, 6 y 8) con el segmento de NS3 mutado y que expresan la proteína VP2 de BTV-8. Tras el desafío de estas ovejasa las tres semanas post-vacunación con una cepa virulenta de BTV-8, se observó que estaban completamente protegidas frente a la infección (Feenstra et al., 2014). El siguiente paso fue la generación de vacunas DISA basadas en BTV-6 que expresan distintas proteínas VP2 (BTV-1, 2, 4, 8 y 9) e incluso que expresan VP2 quiméricas (BTV-1 y BTV-16) (Feenstra, Pap, and van Rijn, 2015) con el fin de desarrollar vacunas multiserotipo. Otro tipo de vacunas basadas en esta tecnología son las llamadas vacunas "DISC" (*Disabled infectious single-cycle*). Estas partículas virales son solo capaces de llevar a cabo un ciclo de replicación debido a que se ha deleccionado el segmento que codifica la helicasa (VP6), siendo necesario una línea celular que exprese esta proteína de manera estable para rescatar el virus. Se ha utilizado como base BTV-1, y en él se han sustituido los segmentos codificantes de VP2 y VP5 de BTV-1 por los de BTV-2, 4, 8, 10, 13 y 21, y todas estas construcciones fueron testadas en vacas y como cóctel de vacunas en ovejas, protegiendo frente a la infección y generando anticuerpos neutralizantes frente a cada uno de los serotipos (Celma et al., 2013; Matsuo et al., 2011). Otros grupos generaron este tipo de virus basados en genética reversa utilizando como base BTV-1, donde sustituyen de nuevo el segmento correspondiente a VP2 y mantienen el segmento codificante de la helicasa. Pero a diferencia de los anteriores, lo inoculan previa inactivación. Tiene la ventaja de ser un método rápido de respuesta frente a brotes de BTV. Se

ha probado con éxito en ovejas y se está escalando su producción a nivel industrial (Nunes et al., 2014).

Otra estrategia vacunal es la basada en el uso de vectores virales como los adenovirus o los poxvirus. Ambos tienen un genoma compuesto por DNA de doble cadena y se consideran seguros como vectores vacunales ya que evitan el riesgo de reorganización génica y de reversión a la virulencia (Alpar, 2009; Fougeroux and Holst, 2017). Son altamente inmunogénicos, activando tanto la respuesta inmune humoral como celular, por lo que son una buena herramienta para inducir protección cruzada. Muchos son los ejemplos de vacunas basadas en estos vectores frente a diversas infecciones producidas tanto por virus, como bacterias o parásitos (Batra et al., 2017; Draper et al., 2009; Pastoret and Brochier, 1996; Volz et al., 2015) e incluso para combatir el cáncer (Wolf et al., 2004; Zhang and Zhou, 2016). Se han desarrollado varios prototipos de vacunas frente a BTV utilizando poxvirus como vector. Por ejemplo se ha observado que un virus vaccinia recombinante de la cepa "Western Reserve", que expresa simultáneamente VP2 y VP5 del serotipo 1 australiano indujo títulos variables de anticuerpos neutralizantes en ovejas, protegiendo parcialmente a los animales en un desafío con BTV-1 (Lobato et al., 1997), aunque esta aproximación de vacuna no ha seguido adelante. Una vacuna basada en virus canarypox recombinantes que expresan VP2/VP5 ha sido descrita recientemente induciendo una alta y efectiva inmunidad protectora en ovejas (Boone et al., 2007), aunque siendo específica frente al serotipo 17 de BTV. En otro estudio se determinó que la vacunación de ovejas con virus capripox recombinante que expresa VP7 de BTV confiere una protección heterotípica parcial frente a otros serotipos del virus de la lengua azul (Wade-Evans et al., 1996), ya que la mayoría de animales vacunados se recuperó de la infección, aunque todos mostraron signos clínicos. Además, se han vacunado cabras con virus capripox recombinantes que expresan individualmente VP2, VP7, NS1 y NS3 de BTV-2 consiguiendo una prevención total de la viremia tras el desafío con el serotipo homólogo tres semanas después de la aplicación combinada, pero la eficacia en ovejas fue mucho menor (Perrin et al., 2007). La replicación defectiva del adenovirus canino que expresa VP7 de BTV-2, indujo una buena respuesta humoral aunque no neutralizante, siendo solo capaz de proteger parcialmente frente a un desafío homólogo y no frente al heterólogo con BTV-8 (Bouet-Cararo et al., 2014).

En nuestro laboratorio se analizó la eficacia de una estrategia de vacunación *prime-boost* basada en una dosis de DNA y una segunda dosis con MVA que expresaban las proteínas VP2, VP5 y VP7 de BTV-4 en ratones IFNAR(-/-), observándose una eficacia del 100% en animales desfiados con el virus homólogo, mientras que la protección frente al desafío

heterólogo fue parcial (Calvo-Pinilla et al., 2009b). Con el fin de mejorar la eficacia en cuanto a cross-protección, en otro ensayo se generó un DNA y un MVA que expresaban la proteína NS1, debido a que se ha descrito que las proteínas no estructurales, sobretodo NS1 y NS2, son las mayores inductoras de respuesta inmune celular, muy relacionada con la protección multiserotipo. En combinación con los cDNAs y MVAs que expresaban VP2 y VP7 se consiguió una protección total tanto frente al serotipo homólogo como a serotipos heterólogos (Calvo-Pinilla et al., 2012). El desarrollo de vectores vacunales que expresen antígenos inmunodominantes conservados entre serotipos y que aumenten su presentación al sistema inmune podría reducir el número de vacunaciones individuales frente a los distintos serotipos, y así generar una vacuna más barata, efectiva y de amplio espectro.

9. *El ratón IFNAR(-/-) como modelo animal de laboratorio para estudios de patología e inmunización.*

Tradicionalmente, para el estudio de todos los aspectos biológicos que rodean a BTV, como su biología, patogenicidad, la interacción entre el virus y el sistema inmune o los ensayos de vacunación se ha utilizado el hospedador natural, generalmente la oveja, aunque también en menos grado vacas y cabras. La dificultad que entraña el manejo de estos grandes animales, así como los costes derivados de su mantenimiento o la necesidad de disponer de grandes instalaciones, que en el caso de BTV han de ser de nivel 3 de bioseguridad, han dificultado y limitado todos estos estudios. Para solucionar estos inconvenientes se ha optado por buscar un modelo animal de laboratorio, más pequeño y manejable, que reduzca costes y que permita agilizar los experimentos. Un buen modelo es el ratón, por su fácil manejo, velocidad de crecimiento y madurez, no requieren grandes instalaciones y se pueden diseñar experimentos con un mayor numéro de individuos. Es problema es que los ratones adultos no son susceptibles a la infección por BTV aunque si lo son los ratones neonatos (Brewer and Osburn, 1998; Carr, Brewer, and Osburn, 1995; Letchworth and Appleton, 1983).

En nuestro laboratorio, se propuso un modelo de ratón knockout deficiente en la subunidad β del receptor de IFN tipo I, el ratón IFN-α/β IFNAR(-/-) y se caracterizó su utilidad para los estudios de respuesta inmune y eficacia de vacunas frente a BTV (Calvo-Pinilla et al., 2009a). Puesto que BTV es un potente inductor de IFNα en el hospedador natural (Jameson, Schoenherr, and Grossberg, 1978) y además se ha descrito que el IFNα juega un papel esencial en alcanzar el estado antiviral (picos altos de concentración de IFNα reducen la viremia hasta un 90%) (Foster et al., 1991), los ratones IFN-α/β IFNAR(-/-) fueron susceptibles y altamente

sensibles a la infección por BTV, a pesar de mantener el resto del sitema inune prácticamente intacto (Fiette et al., 1995). Este modelo también ha sido usado para el estudio de la respuesta immune y patología de otros virus como los coronavirus, vaccinia, sarampión, virus de la fiebre del Valle del Rift y virus del Nilo Occidental (Bouloy et al., 2001; Ohka et al., 2007; Ohno et al., 2007; Schuessler et al., 2012; Waibler et al., 2007). En el caso de BTV, se ha descrito la susceptibilidad de estos ratones a la infección con varios serotipos de BTV (BTV-1, BTV-4 o BTV-8). Se ha observado que el virus replica en los mismos órganos diana que en el hospedador natural como bazo, pulmón, timo y linfonodos, reproduciendo lo que ocurre en rumiantes. Además, se ha confirmado la idoneidad de este modelo de ratón para el estudio de eficacia de vacunas(Calvo-Pinilla et al., 2009a; Ortego, de la Poza, and Marin-Lopez, 2014), siendo actualmente utilizado por algunas empresas farmacéuticas punteras en sanidad animal para los estudios de potencia de sus vacunas antes de salir al mercado.

OBJETIVOS

El objetivo general de esta tesis es el desarrollo de vacunas recombinantes marcadoras y estrategias vacunales que confieran protección multiserotipo frente a la infección de BTV. Los objetivos específicos abordados en este trabajo fueron los siguientes:

1- Estudiar si la infección de BTV el modelo de ratón IFNAR (-/-) reproduce los aspectos patológicos descritos en rumiantes infectados con este virus.

2- Generar vacunas subunidad particuladas frente a BTV basadas en microesferas de la proteína muNS-Mi de reovirus aviar que contengan las proteínas VP2, VP7 y NS1 de BTV y que induzcan protección en ausencia de adyuvante.

3- Desarrollar estrategias combinadas de vacunación, basadas en microesferas y el vector viral vacunal MVA que generen protección frente a múltiples serotipos de BTV.

4- Estudiar la capacidad de la proteína no estructural NS1 de BTV, conservada entre los 27 serotipos descritos, como antígeno inductor de respuesta celular y protección multiserotipo frente al virus.

CAPÍTULOS

CAPÍTULO I: **Estudio de la patología producida por la infección de BTV el modelo de ratón IFNAR (-/-)**.

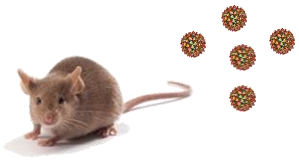
CAPÍTULO II: **Desarrollo de estrategias combinadas de vacunación multiserotipo frente a BTV basadas en microesferas de la proteína muNS-Mi de reovirus aviar y el vector viral MVA que expresen los antígenos VP2, VP7 y NS1.**

CAPÍTULO III: **Estudio de la capacidad de la proteína no estructural NS1 de BTV, conservada entre los 27 serotipos descritos, como antígeno inductor de respuesta celular y protección multiserotipo frente al virus.**

Estudio de la patología producida por la infección de BTV el modelo de ratón IFNAR (-/-).

La lengua azul es una enfermedad hemorrágica causada por un arbovirus, el virus de la lengua azul, que afecta a rumiantes y es transmitida por insectos del género *Culicoides.* BTV es capaz de replicar en tejidos linfoides, infectando leucocitos mononucleares, los cuales secretan mediadores proinflamatorios (citoquinas) y vasoactivos que pueden contribuir al agravamiento de la patogénesis producida por el virus. En este trabajo analizamos la histopatología y la dinámica de las poblaciones leucocitarias en diferentes órganos diana (bazo, timo y pulmón) durante la infección por BTV-4 mediante técnicas histológicas e inmunohistoquímicas en el modelo de ratón IFNAR(-/-), previamente caracterizado en nuestro laboratorio y ampliamente utilizado como modelo de laboratorio para el estudio de la interacción entre BTV y su hospedador. Tanto el bazo como el timo de los animales infectados mostraron una depleción linfoide severa, observado en cortes de tejidos teñidos con hematoxilina-eosina (H&E) y confirmado mediante inmunohistoquímica, donde se observó una reducción de linfocitos T, utilizando un anticuerpo anti CD3, tanto en timo como en los restos de la pulpa blanca del bazo, puesto que la infección destruye su morfología. También se observó una reducción de la señal frente a CD79, marcador de linfocitos B y un aumento de la señal específica de MAC387, marcador de monocitos/macrófagos, en bazo. La infección por BTV también fue capaz de inducir un aumento en la expresión de la actividad caspasa 3 en el bazo, donde se pudieron observar grandes acúmulos de debrís apoptótico por H&E, así como un incremento sustancial en la inmunoreactividad frente a iNOS, asociado a áreas necróticas de la pula blanca, siendo menos perceptible en timo y pulmón. La inducción de citoquinas proinflamatorias en los órganos donde replica BTV fue evaluada por RT-qPCR. La infección aumentó los niveles de transcripción de los RNA mensajeros de IFN-γ, TNF, IL-6, IL-12-p40 e IL-1β en timo, bazo y pulmón, correlacionando con los niveles de replicación del virus en dichos órganos. Por último se analizaron los niveles de linfocitos, neutrófilos, monocitos y plaquetas en sangre de animales infectados con BTV observándose un aumento en el nivel de neutrófilos y una disminución en el nivel de linfocitos, monocitos y plaquetas respecto a los observado en sangre de ratones no infectados, resultado que reproduce lo observado en la infección de BTV en rumiantes. Con estos resultados se puede afirmar que la progresión de la enfermedad y la patogénesis en el modelo de ratón IFNAR(-/-) simula fielmente en algunos aspectos a la observada durante la infección natural de BTV en rumiante, por lo que este modelo de animal

de laboratorio es una buena elección para progresar en el estudio de la patología, inmunología y en el análisis de la potencia de nuevas vacunas frente a diferentes orbivirus.

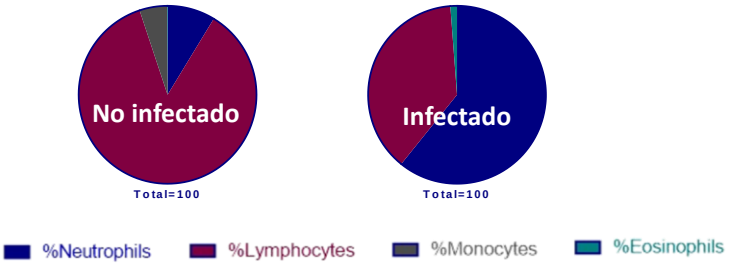


BTV4 M

Sangre

Viremia

Perfil hematológico:
neutrófilos, linfocitos, monocitos y plaquetas

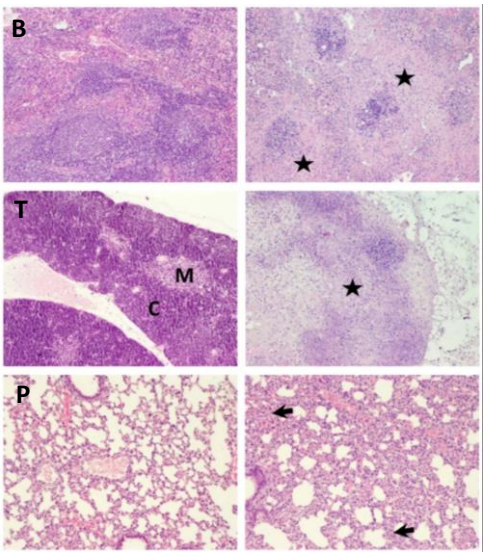


Bazo Timo Pulmón

IFNγ TNFα IL-1β IL-6 IL12p40

Inmunohistoquímica: células B, T y monocitos/macrófagos

No infectado **Infectado**



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Research Paper

Pathological Characterization Of IFNAR(-/-) Mice Infected With Bluetongue Virus Serotype 4

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Abstract

Bluetongue virus (BTV) replicates in lymphoid tissues where infected mononuclear leukocytes secrete proinflammatory and vasoactive mediators that can contribute to bluetongue (BT) pathogenesis. Using the well-characterized IFNAR(-/-) mice animal model, we have now studied the histopathology and dynamics of leukocyte populations in different target tissues (spleen, thymus, and lung) during BTV-4 infection by histological and immunohistochemical techniques. The spleen and thymus of BTV-4 infected mice showed severe lymphoid depletion on H&E stained sections. This finding was confirmed by IHC, showing moderate decreased immunopositivity against CD3 in the thymus, and scarce immunoreactivity against CD3 and CD79 in the rest of the white pulp in the spleen, together with an increase in MAC387 immunostaining. BTV-4 infection also induced the expression of active caspase-3 in the spleen, where apoptotic debris was observed by H&E. A dramatic increase in iNOS immunoreactivity associated to necrotic areas of the white pulp was observed, being less noticeable in the thymus and the lung. The induction of pro-inflammatory cytokines in tissues where BTV replicates was evaluated by measuring transcript levels by RT-qPCR. BTV-4 infection led to enhance transcription of IFN-γ, TNF, IL-6, IL-12-p40, and IL-1β mRNA in the thymus, spleen and lung, correlating with the level of virus replication in these tissues. Disease progression and pathogenesis in IFNAR(-/-) mice closely mimics hallmarks of bluetongue disease in ruminants. IFNAR(-/-) mice are a good choice to facilitate a faster advance in the field of orbiviruses.

Key words: bluetongue virus, IFNAR(-/-) mice, leukocyte, cytokine, pathology.

Introduction

Bluetongue virus (BTV) causes bluetongue, a major hemorrhagic disease of ruminants. The virus, consisting of 27 different serotypes [1-3], is transmitted to its vertebrate host by a few species of biting midges of the *Culicoides* genus (*Diptera*: *Ceratopogonidae*) [4]. Natural BTV infection of ruminants begins with intra-dermal introduction of the virus during the blood feeding of an infected insect. The inoculated virus first infects mononuclear inflammatory cells, including dendritic cells, and it then drains to the local lymph node where further replication occurs prior to the infected cells migrating to the tissues that have been described as the secondary sites of BTV replication such as the lungs, spleen, thymus, and lymph nodes [5-9].

Virus replication in mononuclear phagocytic, dendritic, and endothelial cells (ECs) and the response of these cells to the infection are critical in the pathogenesis of BTV [8, 10-12]. The inflammatory and vasoactive mediators released by BTV-infected ruminants have been proposed as responsible of the increased vascular permeability that characterizes severe BT; specifically, vasoactive mediators released from BTV-infected cells exert paracrine effects that increase the permeability of adjacent blood vessels [13]. Experimental findings have shown that

pro-inflammatory cytokines such as IL-12, IFN-γ, IL-8, and TNF, as well as vasoactive mediators produced in monocytes/macrophages and cytotoxic lymphocytes (CTLs) are likely involved in BTV pathogenesis and viral kinetics [14]. Thus, the endothelial injury observed during BTV infection could be due to the direct pathogenic effect of BTV infection on endothelial cells or to a response to inflammatory mediators released by virus-infected cells. Similarly, production of pro-inflammatory cytokines by macrophages has been shown to contribute to the pathogenesis of human viral hemorrhagic fevers [15-17].

To date, few *in vivo* studies have been carried out to clarify the source and the role of pro-inflammatory cytokines in the appearance of lesions during BTV infection using tissue samples from naturally or experimentally infected ruminants [18]. Because BTV natural hosts are expensive and require specialized animal facilities with biosafety level 3, the IFNAR(-/-) mouse is a good model to study many aspects of pathogenesis, virulence, vaccine efficacy and immune response to BTV [19-26]. In this animal model, we can closely follow infection and disease progression in a short period of time.

The main objective of this work was to study in the IFNAR(-/-) mice model several pathological aspects reported in BTV infected ruminants. The histopathology and dynamics of monocytes/ macrophages, and B and T lymphocytes of infected mice were analyzed by means of histopathological and immunohistochemical methods on tissue samples from IFNAR(-/-) mice experimentally infected with BTV-4. Moreover, the presence of inducible nitric oxide synthase (iNOS), that modulates adaptive immune responses, and active caspase-3, an indicator of apoptosis, were also analyzed in these tissues by immunohistochemistry. This study was completed with the experimental analysis of the induction of expression of pro-inflammatory cytokines (IL-1β, IL-12, IFN-γ, IL-6, and TNF) in the infected tissues to better understand the pathology of BTV.

Materials and Methods

Virus and cells

KC cells, obtained from *C. sonorensis* larvae, were grown in Schneider's insect medium supplemented with 10% FBS. BTV serotype 4 (MOR2009/09) (BTV-4(M)), isolated from sheep blood in KC insect cells [27], was used in the experiments. KC cells and BTV-4(M) were generously provided by Professor Peter Mertens (The Pirbright Institute, UK).

Mice

IFN α/βR$^{o/o}$ IFNAR(-/-) 129/Sv mice and wild type 129/Sv mice were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout the study. Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal, INIA, Madrid, for 1 week before use. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the Centro de Investigación en Sanidad Animal of the Instituto Nacional de Investigaciones Agrarias (Permit number: CEEA 2010-034) . All efforts were made to minimize suffering as well as the number of animals employed.

Animal inoculation and processing of samples

Mice were infected subcutaneously with different doses of virus. Mice were examined for clinical signs daily. Whole blood was collected in EDTA from all animals at regular intervals after inoculation. At varying times post-infection, several mice were sacrificed, perfused with phosphate-buffered saline (PBS), and several organs (spleen, lung, and thymus, and lymph nodes) were harvested. Tissues were homogenized in PBS using a Tissue Lyser homogenizer (Qiagen). The viruses were released from whole blood and homogenized tissues by three freeze/thaw cycles. The amount of infectious virus was measured by real-time RT-qPCR specific for BTV segment 5 as described by Toussaint et al. (2007).

Blood measurements

A multiparameter, Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used to determine the total and differential cell counts in blood collected into EDTA tubes.

Histopathology and immunohistochemistry

Samples from different tissues and organs (spleen, lung, and thymus) were fixed in 10% buffered formalin (pH 7.2) for histopathological studies. After fixation, samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. Sections of 4 µm thick from paraffin wax blocks were cut and stained with hematoxylin and eosin (H & E) for histopathological analyses.

For immunohistochemical procedures, 3 µm thick sections were mounted on slides coated with silane and dried at room temperature (RT) for 24 h. Unless otherwise stated, all incubations were performed at RT in a humid chamber and the slides were washed three times for 5 min in 0.1 M PBS

containing 0.05% Tween-20 (Sigma-Aldrich) after each incubation. The endogenous peroxidase activity was inhibited by incubating sections with peroxidase blocking reagent (Dako, Denmark) for 1 h. The primary antibodies and immunohistochemical procedures are listed in Table 1. After incubation with the primary antisera, sections were incubated for 30 min with the secondary antibody, either anti-rabbit or mouse En Vision+ System Labelled Polymer-HRP (Dako). Peroxidase reaction was developed using 3.3′diaminobenzidine tetrahydrochloride as chromogen diluted 1:50 in a specific buffer (Dako, Denmark). Finally, sections were counterstained with hematoxylin, dehydrated and coverslipped with DePex mounting medium. Negative controls were obtained substituting the primary antibody by an irrelevant antibody or by PBS. Suitable tissues from other species were employed as positive controls.

Total RNA extraction and first-strand cDNA preparation

Spleen, lung and thymus were collected from each mouse and stored in RNAlater® (Ambion, USA) at −80 ºC before use. Organs were homogenized in Trizol® reagent (Life Technologies, USA) using a Tissue Lyzer homogenizer (Qiagen) following the manufacturer´s protocol. Briefly, after Trizol reagent addition and homogenization, chloroform was added. The sample was centrifuged and the aqueous phase was recovered. RNA was precipitated from the aqueous phase with 2-propanol, washed with 75% ethanol, and resuspended in sterile water. RNA concentration and purity were estimated spectrophotometrically based on absorbance at 260 and 280 nm. First-strand cDNA was synthesized from 0.5 µg of total RNA, which was reversely transcribed by using superscript III reverse transcriptase (Life Technologies, USA) and oligo (dT) as a primer.

Analysis of cytokine gene expression and transcriptional regulation

In order to monitor pro-inflammatory cytokines (IL 1-β, IL 6, IL 12p40, IFNγ and TNF) transcript levels in organs (spleen, lung and thymus) of IFNAR(-/-) mice infected with a lethal dose of BTV-4 were quantified by RT-qPCR (ECO™ Real-Time PCR System, Illumina®, San Diego, California, U.S.). PCR experiments were carried out using the first-strand cDNAs as templates with specific primer pairs (Table 2). Target gene copy numbers were normalized using actin gene as a control, also amplified by RT-PCR. RT-qPCR was performed on an Illumina® ECO™ thermal cycler (San Diego, California, U.S.), using SsoFast™ EvaGreen® Supermix (Bio-RadINK, Hercules, California, US) in 10-µL reaction volumes comprising 1 µL of cDNA template, 5 µL of 2x SsoFast™ mixture (with EvaGreen Dye), 1 µL of primer mix (5 µM/each), and 3 µL ddH_2O. PCR conditions were as follows: initial denaturation at 95°C for 30 s followed by 40 cycles of 95°C for 5 s, 60°C for 10 s and 60ºC for 10 s. After PCR, a melting curve analysis was performed by increasing the temperature from 60ºC to 95ºC, with a temperature transition of 0.5ºC/s. Each gene sample was run in duplicate for both infected and negative controls. No template controls were used in all analyses. The software used to analyze the data was EcoStudy®. A dissociation curve was generated for each sample after completion of amplification and analyzed compared with the negative controls. The comparative CT ($2^{-\Delta\Delta}$CT) method was used to analyze variations in gene expression in terms of relative fold differences between the experimental (BTV infected) and calibrator (un-infected control) samples [28]. For relative quantification, the amount of target was normalized to an endogenous reference (actin, housekeeping gene) and the calibrator $2^{-\Delta\Delta}$CT formula was used: where $^{\Delta\Delta}CT = {}^{\Delta}CT$ (sample) - $^{\Delta}CT$ (calibrator), the CT value indicated fractional cycle number at which the amount of amplified target reached the fixed threshold, and $^{\Delta}CT$ represented CT of the target gene subtracted from CT of the housekeeping gene. The mean CT values of duplicate samples of test and control mice were used for analysis. Results were presented as means ± standard error (SE) of data from duplicate replicates.

Table 1. Primary antibodies and immunohistochemical procedures used in the histopathological studies.

Primary antibody	Type	Antigen retrieval method	Working dilution	Incubation parameters
CD3 (IS503, Dako)	Rabbit polyclonal	Heating under pressure in 10 mM Tris-EDTA buffer (pH 9), 15 min	1:600	2 h at RT
iNOS (AHP477, Serotec)	Rabbit polyclonal	None	1:3000	2 h at RT
MAC387 (MS-148-R7, Neomarkers)	Mouse monoclonal	Proteinase K, 1:500 / 15 min	RTU	2 h at RT
Active Caspase-3 (G7481, Promega)	Rabbit polyclonal	Heating under pressure in 1 mM EDTA buffer (pH 8), 2 min	1:500	2 h at RT
CD79α (IR621, Dako)	Mouse monoclonal	Heating under pressure in target retrieval solution high pH (Dako), 15 min	RTU	2 h at RT

Table 2. Primers used to analyze cytokine gene expression by RT-qPCR.

gene	Fwd PRIMER seq (length)	Rev PRIMER seq (length)	Length product (pb)	Anneal temperature (°C)	Primer concentration (µM)	Product Tm (°C)
Actin	GACGATGCTCCCCGGGCTGTATTC (24)	TCTCTTGCTCTGGGCCTCGTCACC (24)	117	60	5	87
IL-1-β	CAACCAACAAGTGATATTCTCCATG (25)	GATCCACACTCTCCAGCTGCA (21)	152	60	5	83
IL-6	ACCACGGCCTTCCCTACTTCAC (22)	TCCTCATTTCCACGATTTCCCAG (23)	134	60	5	81
IL-12p40	ACAGCACCAGCTTCTTCATCA (21)	TCTTCAAAGGCTTCATCTGCA (21)	75	60	5	82
IFNγ	GCGTCATTGAATCACACC (18)	GGACCTGTGGGTTGTTGACC (20)	105	60	5	81
TNFa	AGCCCACGTCGTAGCAAACCAC (22)	ATCGGCTGGCACCACTAGTTGGT (23)	121	60	5	88

Results

Susceptibility of IFNAR(-/-) mice to BTV-4 Morocco strain

To determine the susceptibility of IFNAR(-/-) mice to BTV-4(M), animals were inoculated subcutaneously with ten-fold serial dilutions of BTV-4(M). At a low infection dose (10^2 pfu/mice) IFNAR(-/-) mice were susceptible to BTV-4(M) infection, showing disease clinical signs characterized by ruffled hair, ocular discharges, reduced activity, and apathy starting at 48 h.p.i. Disease progression led to animal death and 100% of the infected animals died at 5 d.p.i. Similar results were observed with a dose of 10^3 pfu/mice. Higher infection dose of 10^4 pfu/mice killed 100% of the animals at 72 h.p.i. and the appearance of clinical signs occurred at 24 h.p.i. (Fig. 1).

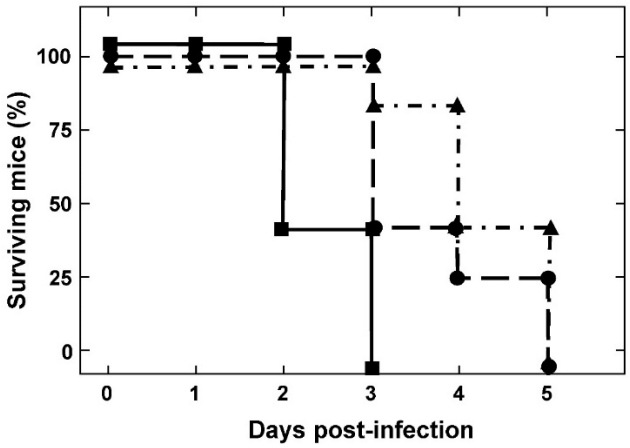


Figure 1. Susceptibility of adult mice to BTV-4 Morocco strain infection. IFNAR(-/-) mice (8 weeks old, 5 mice per group) were subcutaneously inoculated with 10-fold dilutions of BTV-4. The number of PFUs inoculated is indicated on each survival group (10e2 ▲, 10e3●, 10e4■). The mice were observed every 12 h for 5 days.

Blood parameters and viraemia in BTV infected IFNAR(-/-) mice.

Viral spread and viraemia were determined in blood and tissue samples of mice infected with 10^3 pfu/mice by RT-qPCR. Viraemia was detected at 24 h.p.i and the presence of virus in blood was increased thereafter until animal death. The first tissue to be reached by the virus was the spleen where BTV genome was detected as early as 24 h.p.i. (*Ct*: 30.49±1.05) (Table 3). By 48 h.p.i. viral genomes and BTV protein (figure S1) were also detected in the lung, thymus, and inguinal lymph nodes. The presence of BTV genomes increased thereafter until the death of the animal. No infectious virus was detected in the liver, brain, heart, tongue, skin, and testicles at any time points examined (data not shown).

Changes in haematology were determined after infection of IFNAR(-/-) mice with 10^3 pfu/mice (Fig. 2). BTV infection resulted in a significant decrease in the total white blood cell counts by 72 h.p.i (from $9.6x10^6$ to $2.54\ x10^6$ cells/ml) highlighted by a 8-fold drop in the absolute lymphocyte counts as well as a 60% reduction in the percentage of lymphocytes. In addition, a dramatic reduction in the absolute monocyte count was detected in the infected mice. In contrast, an increase in the neutrophil percentage and in their absolute count was observed due to the BTV infection. Platelet counts also decreased in BTV-infected mice by up to 2-fold over the course of the infection. Thrombocytopenia is indicative of coagulation disorders and may partially explain the haemorrhages observed in the spleen, lung and liver of BTV infected mice (figure S2).

Table 3. Detection of BTV-4(M) in blood and tissues of infected IFNAR(-/-) mice after challenge by RT-qPCR_S5.

	24 h.p.i.	48 h.p.i.	72 h.p.i.
Blood	29.07±1.02	25.05±0.23	23.78±1.1
Thymus	Neg.	29.17±21.54	21.71±0.92
Lung	36.69±1.23	22.84±1.1	20.77±3.31
Spleen	30.49±1.05	22.88±2.32	18.89±1.37
Lymph node (inguinal)	N.D.	25.82±1.25	22.37±2.64

Results expressed as *Ct* and transferred to negative (neg.) according to the cut-off *Ct*≥38 described by Toussaint et al. (2007).

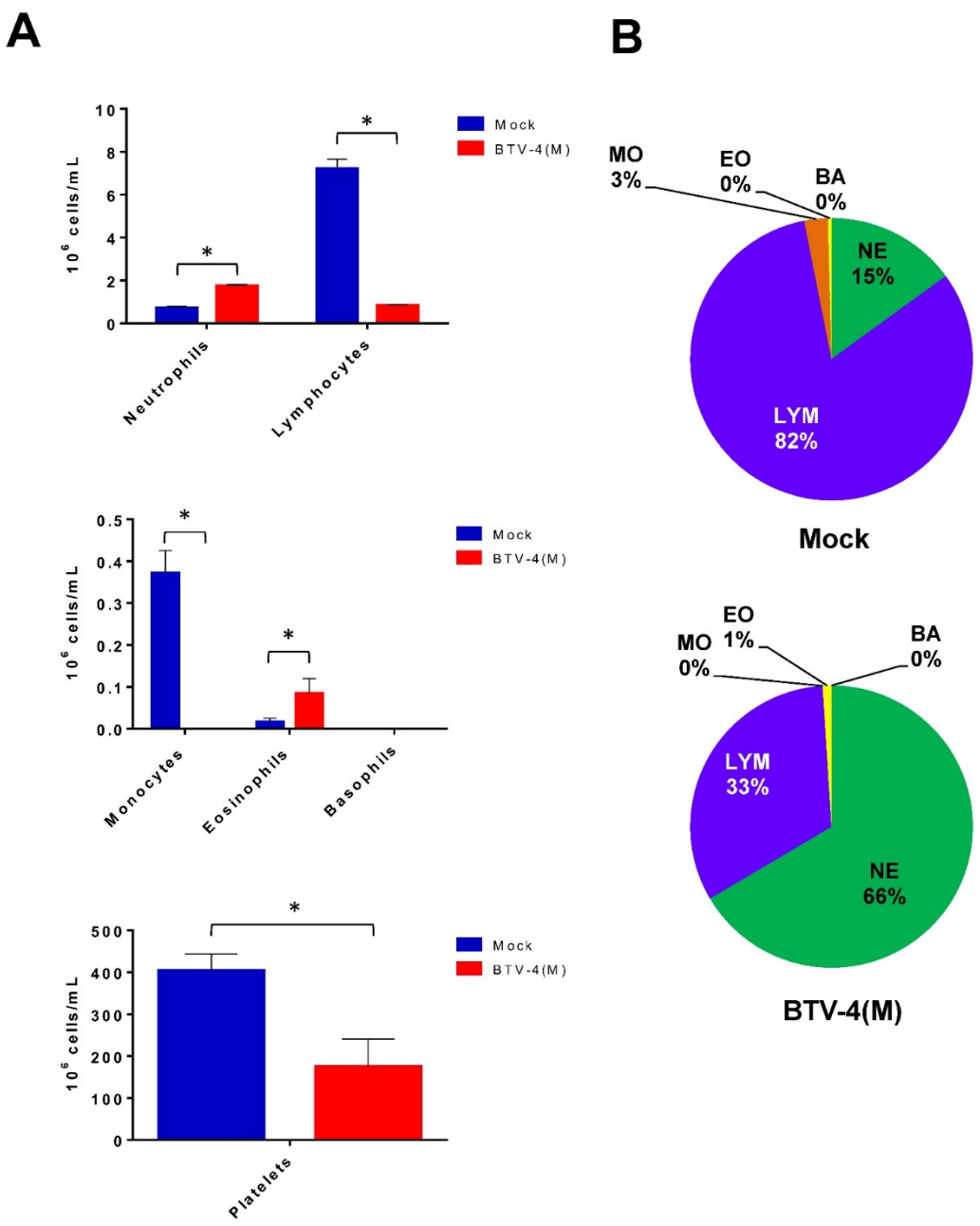


Figure 2. Blood parameters in IFNAR(-/-) mice infected with BTV-4 Morocco strain. Mice were infected subcutaneously with 10^3 PFU of BTV-4 (MOR2009/09) per mouse. Whole blood was collected at 72 h.p.i. and the following parameters were analyzed: lymphocytes, monocytes, neutrophils, basophils, eosinophils, and platelets. (A), Cell count. (B), Cell percentage. The results represent the average of 5 mice ± SD. Asterisks represent significant difference between samples, calculated by Man-Whitney non parametric test ($p \leq 0.01$).

Histopathologic changes in organs of BTV infected IFNAR(-/-) mice

In order to study the pathological effects of the infection in the organs where BTV-4 replicates, histological analysis were performed on several organs extracted from BTV infected and uninfected IFNAR(-/-) mice at 72 h.p.i. Light microscopy on lungs from infected mice revealed diffuse interstitial pneumonia characterized by hyperaemia, thickening of alveolar septa with infiltrates composed by mononuclear cells and scarce neutrophils, and the presence of oedema and leak of red blood cells into the alveolar spaces (Fig. 3). The spleen of infected mice showed severe white pulp lymphocytolisis and lymphoid depletion, as well as neutrophilic infiltrates in the margin between the red and white pulp (Fig. 3). As splenic lesions progressed, the white pulp was reduced to small foci of periarteriolar lymphoid tissue, surrounded by abundant apoptotic debris, neutrophils and siderocytes (Fig. 3). Lymphoid depletion was also observed in the thymus, where the typical structure of medulla-cortex was

indistinguishable and large areas of the parenchyma were replaced by necrotic tissue (Fig.3).

By means of immunohistochemistry, a dramatically decrease of immunoreactivity against CD3 antibody (a T cell marker) was observed in the spleen and thymus of the infected mice, with foci of CD3+ cells in periarteriolar areas of the spleen and few positive cells scattered through the thymus parenchyma (Fig. 4). Similarly, a lower number of cells immunoreactive against CD79 (a B cell marker), restricted to the remnants of the white pulp, was observed in the spleen from infected mice compared to control. Besides, scarce small foci of CD79+ cells were found in the lungs, close to pneumonic areas (Fig. 5). On the other hand, a clear increase in immunoreactivity against MAC387 and iNOS antibodies was detected in the spleen and thymus of the infected mice. Positive cells to both antibodies were diffusely observed in the thymus parenchyma, whereas in the spleen, MAC387+ cells were mainly located in the remaining white pulp (Fig. 6) and iNOS+ cells were not only associated with the white pulp, but also scattered throughout the necrotic areas (Fig. 7). A few number of immunopositive cells against iNOS and MAC387 antibodies were also observed in the mononuclear inflammatory infiltrates in the lung of BTV-infected animals. The morphology of cells positive to MAC387 antibody was compatible with either monocytes/macrophages, whereas iNOS+ cells were compatible with both monocytes/ macrophages and neutrophils. Furthermore, BTV-4 infection also induced a higher expression of active caspase-3 in the spleen and thymus from BTV-infected mice compared to control animals, concurring with the apoptotic debris observed by H&E (Fig. 8). In the remaining organs evaluated, no significant changes were observed between control and infected mice for neither the number nor the distribution of cells immunoreactive against the antibodies employed in this study.

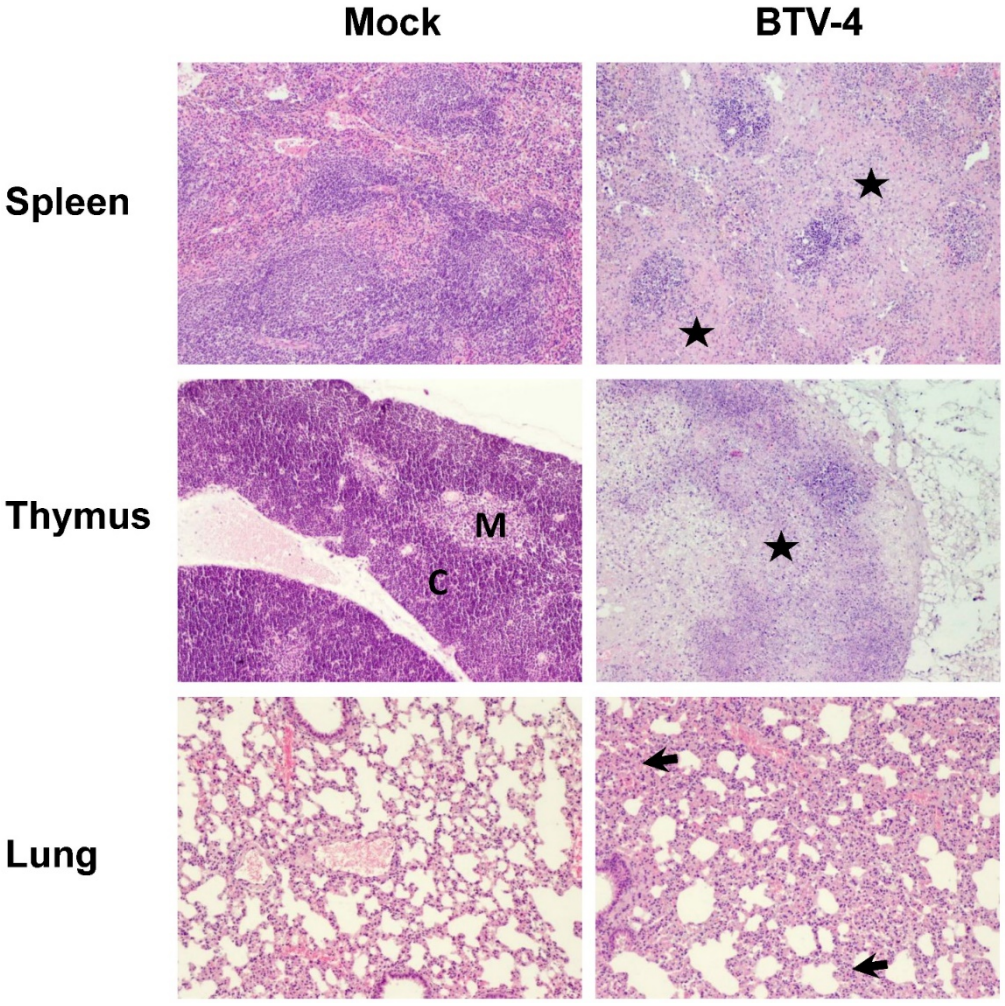


Figure 3. Comparative photomicrograph of tissue sections from BTV-4- infected and control animals. H&E, 100X. IFNAR(-/-) mice (*n* = 3) were infected subcutaneosuly with 10^3 PFU of BTV-4. Representative pictures of tissues from the spleen, thymus, and lung collected at 72 h.p.i. and stained with hematoxylin and eosin, are shown. Extensive necrosis and lymphoid depletion, mainly located in the marginal zone bordering the white pulp (asterisk), was detected in the spleen from BTV-infected specimens, as well as a population of neutrophils and macrophages invading necrotic tissue. The thymus of BTV-infected animals showed extensive necrosis and lymphoid depletion (asterisk), with loss of the boundaries between the medulla (M) and the cortex (C). In the lungs of BTV-infected animals a diffuse interstitial pneumonia was encountered, showing multifocal atelectasis and increased septum size (arrows), with infiltration of mononuclear cells and scarce neutrophils.

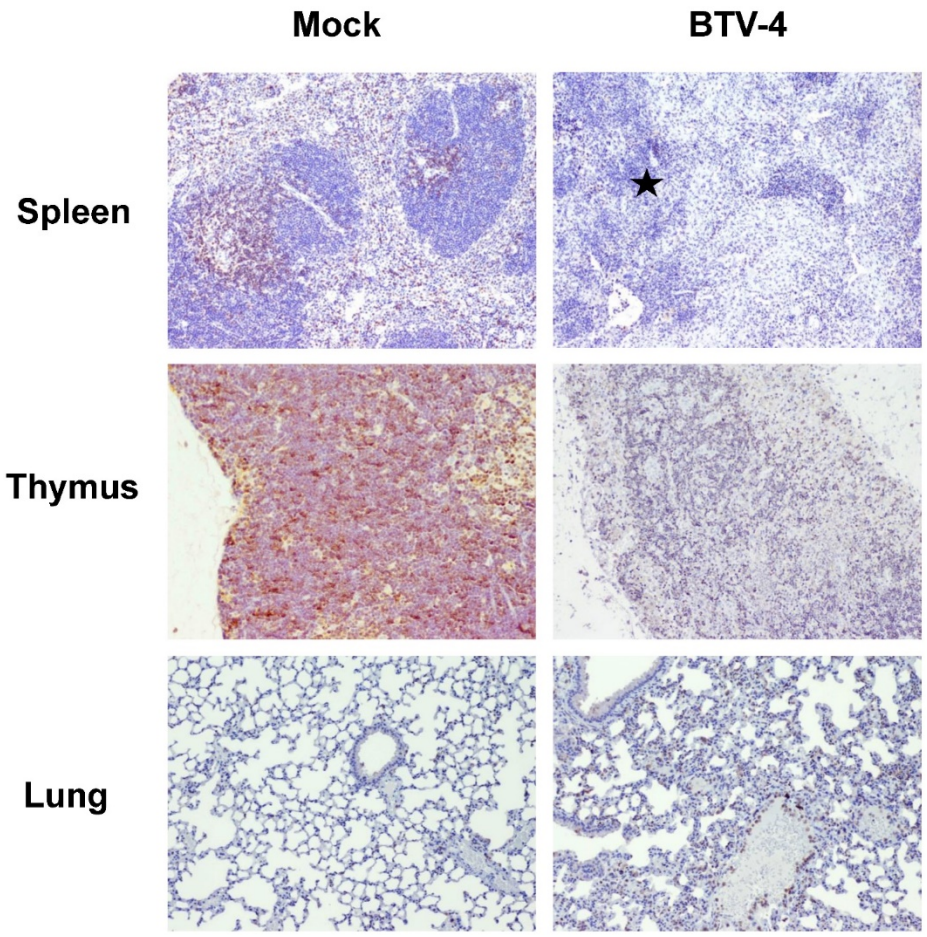


Figure 4. Representative photomicrographs showing the difference in the number/distribution of CD3 positive cells in spleen, thymus and lung, between BTV-infected and Mock mice. IHC against CD3, 100X. A significant decrease in the number of CD3+ cells was observed in spleen and thymus from infected animals. Populations of CD3+ cell were restricted to periarteriolar areas in spleen (asterisk). A mild increase in the number of CD3+ cells, dispersed throughout the pulmonary parenchyma, was also observed.

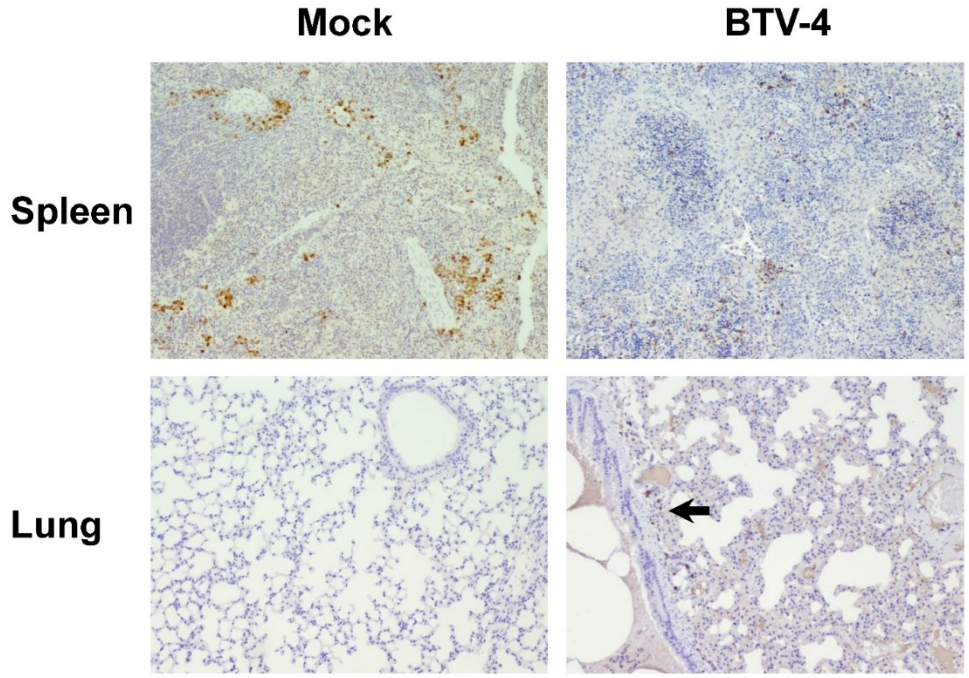


Figure 5. Representative photomicrographs of immunohistochemical technique against CD79 antibody in spleen and lungs from BTV-4-infected and Mock animals. IHC against CD79 antibody, 100X. Immunoreactivity against CD79 was mainly present close to the remnants of the white pulp in the spleen of infected mice, being diminished in BTV-infected mice compared to controls. A small cluster of CD79+ cells (arrow) was observed in lungs of BTV-infected animal, within a pneumonic focus.

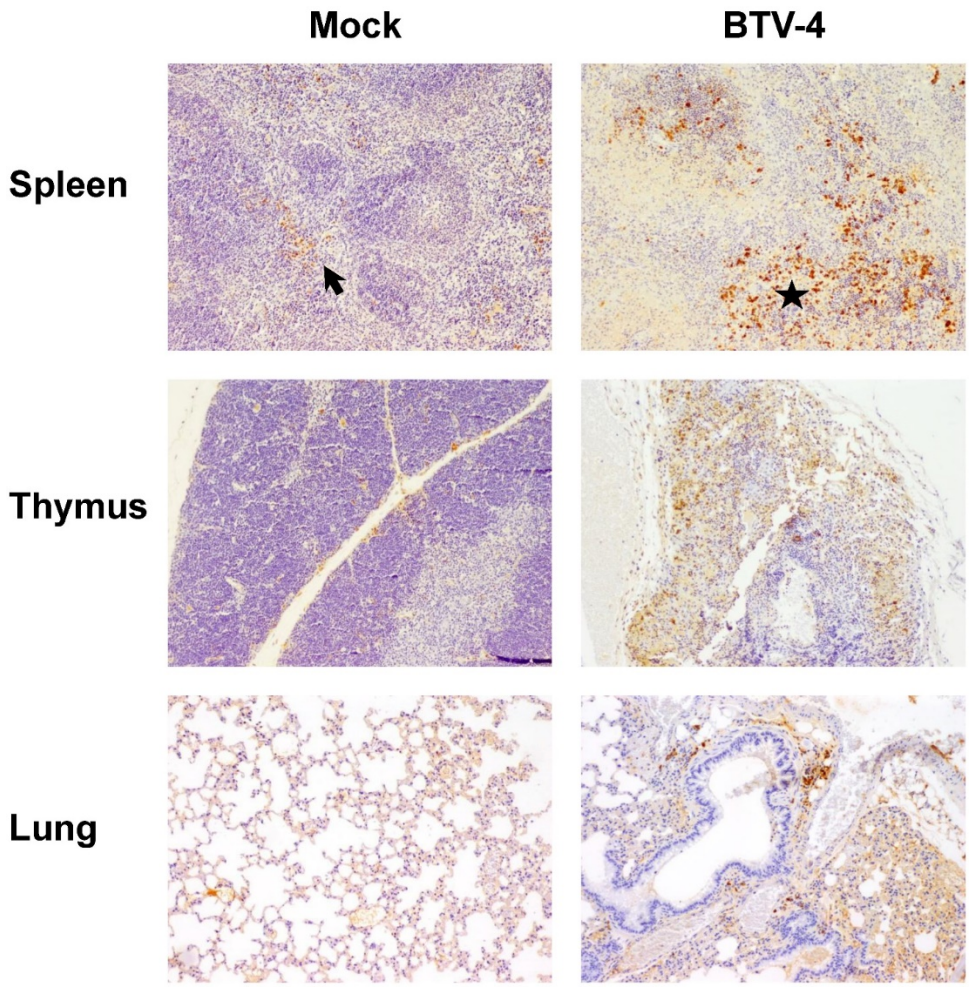


Figure 6. Representative micrographs of immunohistochemical technique against MAC387 antibody in spleen, thymus and lungs from BTV-4-infected and Mock animals. IHC against MAC387 antibody, 100X. Positivity against MAC387 antibody in Mock-infected mice was mainly found in medullar sinuses (arrow), whereas in infected individuals the number of MAC387+ cells was increased, being scattered throughout the white pulp (asterisk). Immunoreactivity against MAC387 antibody was higher in the thymus from BTV-4-infected mice when compared with control animals, mainly in peripheral areas of the thymic parenchyma. Few small foci of MAC387+ cells (arrow) present in lung of BTV-infected animal, close to a bronchiole.

BTV infection causes strong proinflammatory immune responses in IFNAR(-/-) mice

To further investigate the pathogenesis of BTV, we evaluated the induction of pro-inflammatory cytokines in the tissues where BTV replicates by measuring transcript levels. IFNAR(-/-) mice developed strong pro-inflammatory immune responses following BTV-4 infection, as demonstrated by a significant increase in mRNA of IL-1β, IL-6, IL-12p40, IFN-γ, and TNF in the lung, and specially in the thymus and spleen (Fig. 9). The upregulation of pro-inflammatory cytokine mRNA correlated with virus replication, iNOS induction and the presence of monocytes/macrophages in these tissues.

Discussion

Ruminants are the natural host species in which BT disease occurs and are therefore the preferred animals to conduct experimental infection studies. However, infection studies using ruminants are expensive and time consuming and a limited number of animals are generally included [29]. Inbred IFNAR(-/-) mice are far easier and cheaper to work with. A tremendous advantage of this mouse model is the availability of a wide variety of reagents that can be used to study many aspects of the immune response to the virus. Although extrapolation of findings in mice to ruminants must be done with care due to differences in the biology between mouse and ruminant species, IFNAR(-/-) mice have been used as an animal model to study BTV transmission, pathogenesis, virulence, and protective efficacy of inactivated and new recombinant marker BTV vaccines [19-26].

The susceptibility of IFNAR(-/-) mice to BTV infection has been previously characterized in our laboratory for strains of BTV-1, BTV-8, and BTV-4 [20, 30, 31]. For the present work we have used a strain of BTV-4 (MOR2009/09) isolated from sheep blood by

using a culicoides cell line (KC cells) that has not been passed through mammalian cell lines. The clinical disease produced in IFNAR(-/-) mice infected with BTV-4(M) includes high viraemia and viral tissue titers in spleen, lung and thymus, which were detectable starting at 2 d.p.i. until death. Thus, an increased percentage of neutrophils and profound loss of lymphocytes, monocytes and platelets was observed in the blood after BTV infection. These results reproduce the data reported in experimental infections of sheep with BTV-23 and white-tailed deer with BTV-17 where marked thrombocytopenia, lymphopenia, and neutrophilia were observed [13, 32, 33]. Lymphoid depletion and neutrophilic infiltrates were observed in H&E stained sections of spleen and thymus of BTV-4(M) infected IFNAR(-/-) mice. Lymphoid depletion was confirmed by IHC, showing moderate decreased immunopositivity against CD3 in the thymus, and scarce immunoreactivity against CD3 and CD79 in the remains of the white pulp of the spleen. The reduction in CD3 and CD79 reactivity in lymphoid tissues of BTV infected mice indicates lymphopenia, as it has been described commonly in sheep during infections with several BTV serotypes. One of the mechanisms proposed for lymphoid depletion includes apoptosis [34]. Alternatively, BTV replication in lymphocytes, monocytes/macrophages and/or dendritic cells could result in cellular injury [35]. Lymphoid depletion could produce immunosupression and predisposition to secondary microbial infections as it has been observed in experimental infections of sheep with BTV. A positive signal specific of BTV has been observed in the lymph nodes, spleen, and thymus of BTV infected mice [31]. In addition, a clear increase of active caspase-3 was detected in the same tissues after infection indicating that both mechanisms can be also implicated in the lymphoid depletion observed in IFNAR(-/-) mice.

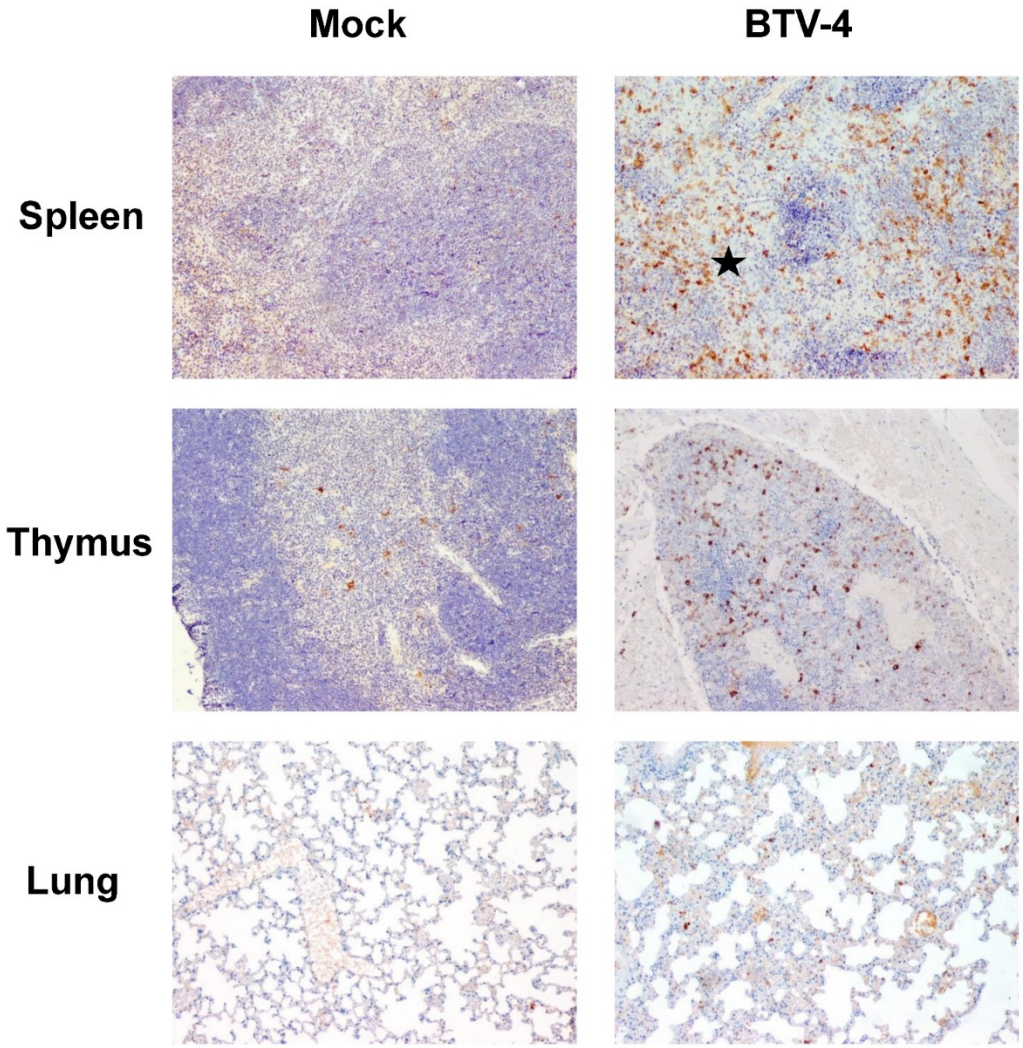


Figure 7. Representative micrographs of immunohistochemical technique against iNOS antibody in spleen, thymus and lungs from BTV-4-infected and Mock animals. IHC against iNOS antibody, 100X. The number of iNOS+ cells was highly increased in spleen from BTV-infected animals and these cells were mainly located in necrotic areas surrounding the white pulp (asterisk). In the thymus of infected mice, immunoreactivity against iNOS antibody was higher than mock-infected individuals and was randomly distributed throughout the parenchyma. A few number of iNOS+ cells was present in the inflammatory infiltrate of the lung of infected mice.

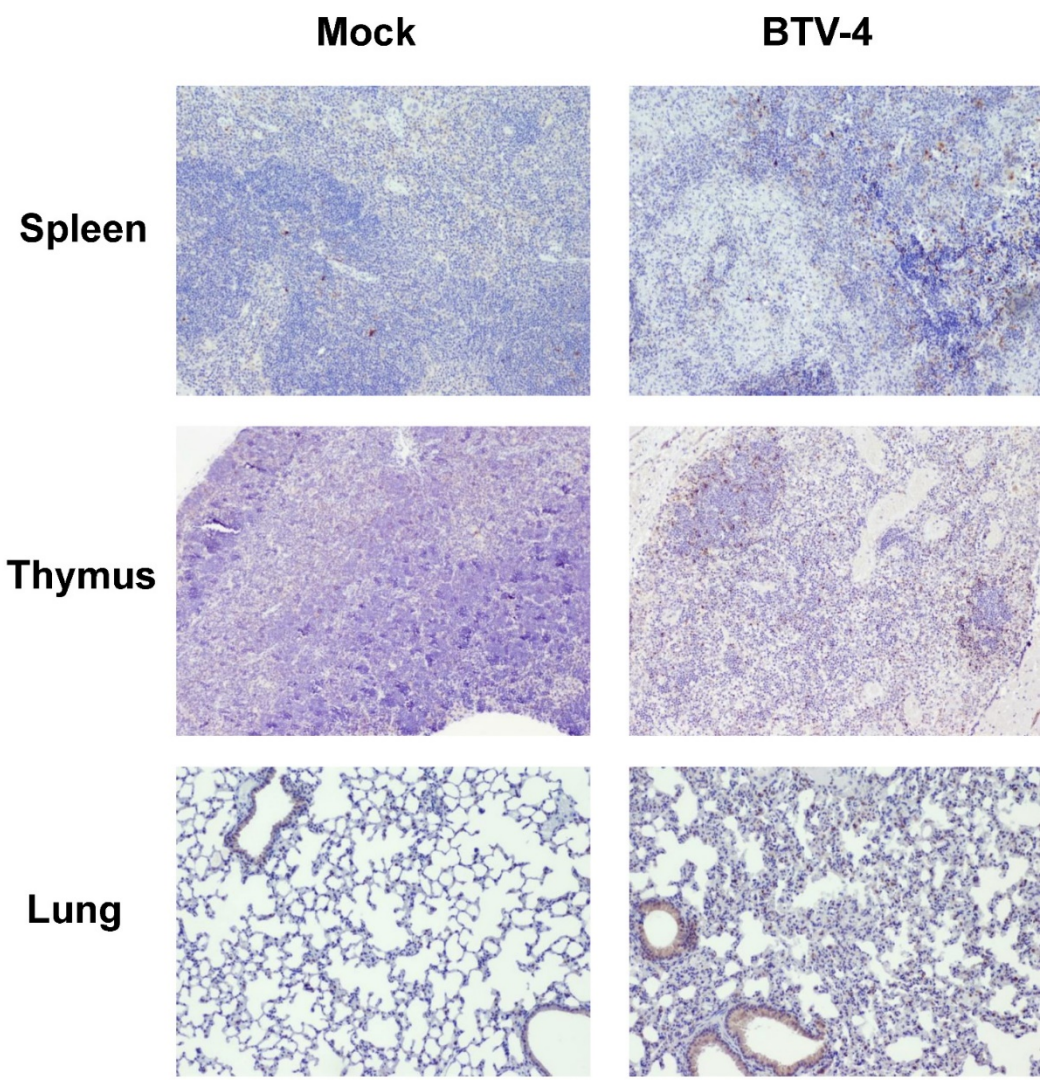
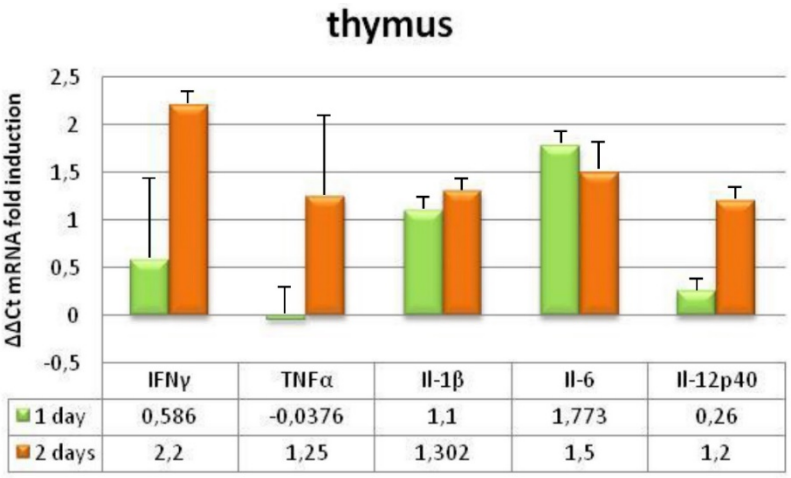


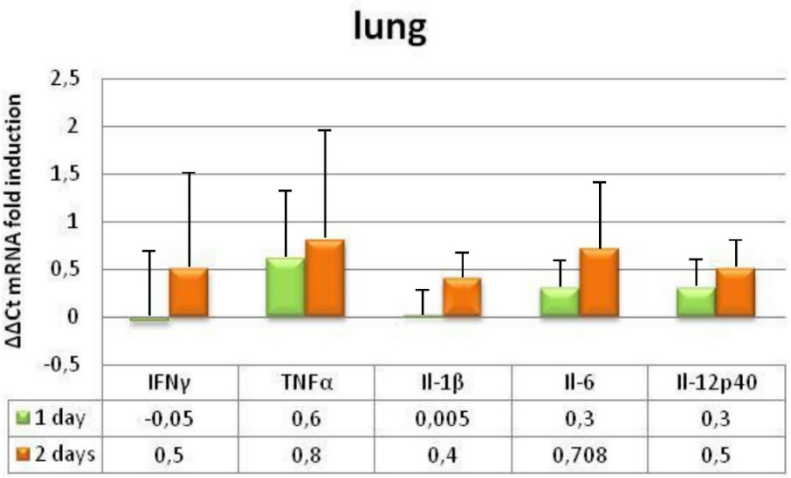
Figure 8. Representative micrographs of immunohistochemical technique against active caspase-3 antibody in spleen, thymus and lung from BTV-4-infected and Mock animals. IHC against caspase-3 antibody, 100X. The number of active caspase-3 cells was moderately increased in spleen, thymus, and lung from BTV-infected animals, concurring with the apoptotic debris.

In contrast, an increase in MAC387 immunostaining was observed mainly in spleen and thymus and, to a lesser extent, in lung of infected mice. The morphology of cells positive to MAC387 antibody was compatible with either monocytes/macrophages. The accumulation of macrophages in these three tissues has been previously reported in BTV infected ruminants [9, 36] and it has been suggested that macrophages are positioned in the infected tissues to capture, internalize and process BTV antigens.

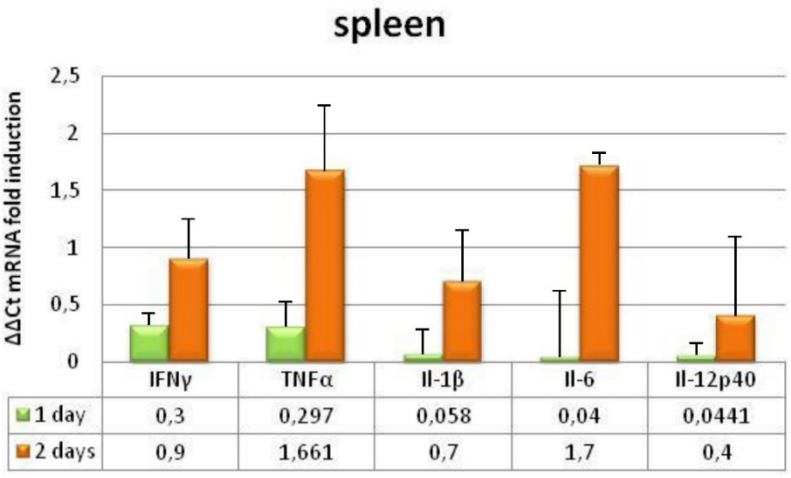
Previously it was reported from both *in vivo* and *in vitro* studies that BTV- infected mononuclear leukocytes (dendritic cells, macrophages, monocytes and some lymphocytes) secrete numerous pro-inflammatory cytokines, including IFN-α, IFN-γ, IL-12, TNF, IL-1β and IL-8 [8, 14, 37-39]. Induction of a balanced antiviral and pro-inflammatory response is crucial to control viral replication and activate optimal adaptive immune responses. Although such response is required for viral clearance, depending on the type of disease, it may also damage tissues in severe cases, contributing to disease pathogenesis [40]. The endothelial injury observed in ruminants during BTV infection could be due to the direct pathogenic effect of BTV infection on endothelial cells or to a response to inflammatory mediators released by virus-infected endothelial cells and, possibly, other cell types such as monocytes/macrophages [8, 14, 18, 39]. Furthermore, *in vitro* BTV infections of peripheral blood mononuclear cells (PBMC) from a number of ruminant species resulted in the production of inflammatory cytokines. BTV infection of PBMC from bovine [39], sheep and goats [41] induced IL-1, 6, 8, and 10, IFN-γ, TNF and iNOS, although these studies used different experimental designs and BTV serotypes, making correlations among species difficult.



	IFNγ	TNFα	Il-1β	Il-6	Il-12p40
1 day	0,586	-0,0376	1,1	1,773	0,26
2 days	2,2	1,25	1,302	1,5	1,2



	IFNγ	TNFα	Il-1β	Il-6	Il-12p40
1 day	-0,05	0,6	0,005	0,3	0,3
2 days	0,5	0,8	0,4	0,708	0,5



	IFNγ	TNFα	Il-1β	Il-6	Il-12p40
1 day	0,3	0,297	0,058	0,04	0,0441
2 days	0,9	1,661	0,7	1,7	0,4

Figure 9. Proinflammatory cytokines changes in IFNAR(-/-) mice infected with BTV-4 Morocco strain. Tissue samples were collected at the indicated time points from individual IFNAR(-/-) mice (3 per time point) infected with 10^3 PFU of BTV-4. Total RNA from spleen, thymus and lungs were extracted, and the expression of mRNA of proinflammatory cytokines was quantified. The expression levels of the different genes were normalized to levels in mock-infected mice. Columns represent mean values and error bars represent the SD of three independent mice per group.

To further investigate whether the presence of macrophages in spleen, thymus, and lung of BTV infected IFNAR(-/-) mice correlated with an activation of pro-inflammatory response in these tissues we evaluated the induction of pro-inflammatory cytokines in the tissues where BTV replicates by measuring transcript levels. BTV infection of IFNAR(-/-) mice resulted in increased transcription of genes encoding IL-1β, IL-6, IL-12p40, IFN-γ, and TNF. In addition, the higher presence of

macrophages in spleen and thymus correlated with higher induction of pro-inflammatory cytokines, tissues, which also showed higher histopathological alterations. Besides the induction of pro-inflammatory cytokines observed in serum and tissues of BTV infected ruminants, it has been also reported that BTV infection of sheep led to enhance transcription of iNOS in peripheral blood mononuclear cells and regional lymphoid organs [42]. Furthermore, in vitro studies provided evidence that cytokines and other vasoactive substances produced in macrophages potentially contributed to vascular injury in BTV-infected ruminants [39]. The IFNAR(-/-) mouse model reproduced *in vivo* the increase expression of iNOS in BTV-4(M) infected tissues. Positive cells to iNOS antibody were slightly augmented in lung and greatly increased in spleen and thymus of infected animals, compared to the control mice, and the morphology of cells positive to iNOS+ were compatible with both monocytes/macrophages and neutrophils. Nitric oxide is a potent anti-viral molecule to combat infection in combination with acute phase proteins and cytokines. Nevertheless, the excessive production of iNOS, and subsequent high levels of NO during viral infections may have negative effects, acting with other damaging oxidants to promote excessive inflammation or induce apoptosis, as it has been described in influenza virus infection [43]. As it has been described in the pathogenesis of viral hemorrhagic fevers [15, 44, 45], the overproduction of iNOS and pro-inflammatory cytokines in BTV infected tissues could contribute to the pathology of this disease-causing vasodilatation, increase of leukocyte adhesion and thrombus formation, and increase of vascular permeability of endothelial cells with subsequent tissue edema [39, 46].

BTV induces apoptosis both in cultured cells and in target tissues *in vivo* and one current hypothesis is that apoptosis plays a major role in the pathogenesis of BTV infection [47-49]. The spleen of BTV infected IFNAR(-/-) mice showed apoptotic debris and high presence of active caspase-3, reproducing the apoptotic effect observed in BTV infected sheep. Active-caspase-3 is an important pro-apoptotic molecule and acts as final effector in all apoptotic pathways, and it can be considered as a good marker for apoptosis [50]. It is noteworthy that the number of positive cells against active caspase-3 is not high enough to explain the massive necrosis and lymphoid depletion that take place in spleen and thymus of BTV-infected mice. This is probably because Caspase-3 is unable to detect all apoptotic cells [51] , and necrosis also occurs in these tissues, likely due to the increased oxidative environment. The role of active caspase-3 in BTV-induced apoptosis has been confirmed in cell lines [47] and in sheep infected with BTV-23 where apoptosis in the spleen and peripheral blood mononuclear cells (PBMC) and up-regulation of caspase-3 mRNA in PBMC were observed [34]. It has been suggested that IFN-α could mediate apoptosis activating the release of pro-inflamatory cytokines and mediating nitric oxide production from activated macrophages or endothelial cells [34]. The induction of apoptosis in the BTV infected IFNAR(-/-) mice, where the IFN-α signaling pathway is blocked, indicates that BTV infection could lead to apoptosis in an independent IFN-α fashion.

Ruminants are currently the best animal model to study BTV infection. Furthermore, any promising vaccine or treatment to be considered for clinical trials must be successful in ruminants. However, due to ethical, practical, and financial considerations, a murine model that reproduces pathological aspects of viral infection is a valuable tool to screen potential vaccine efficacy and to study BTV transmission, pathogenesis and virulence.

Supplementary Material

Supplementary figures.
http://www.ijbs.com/v12p1448s1.pdf

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Competing Interests

The authors have declared that no competing interest exists.

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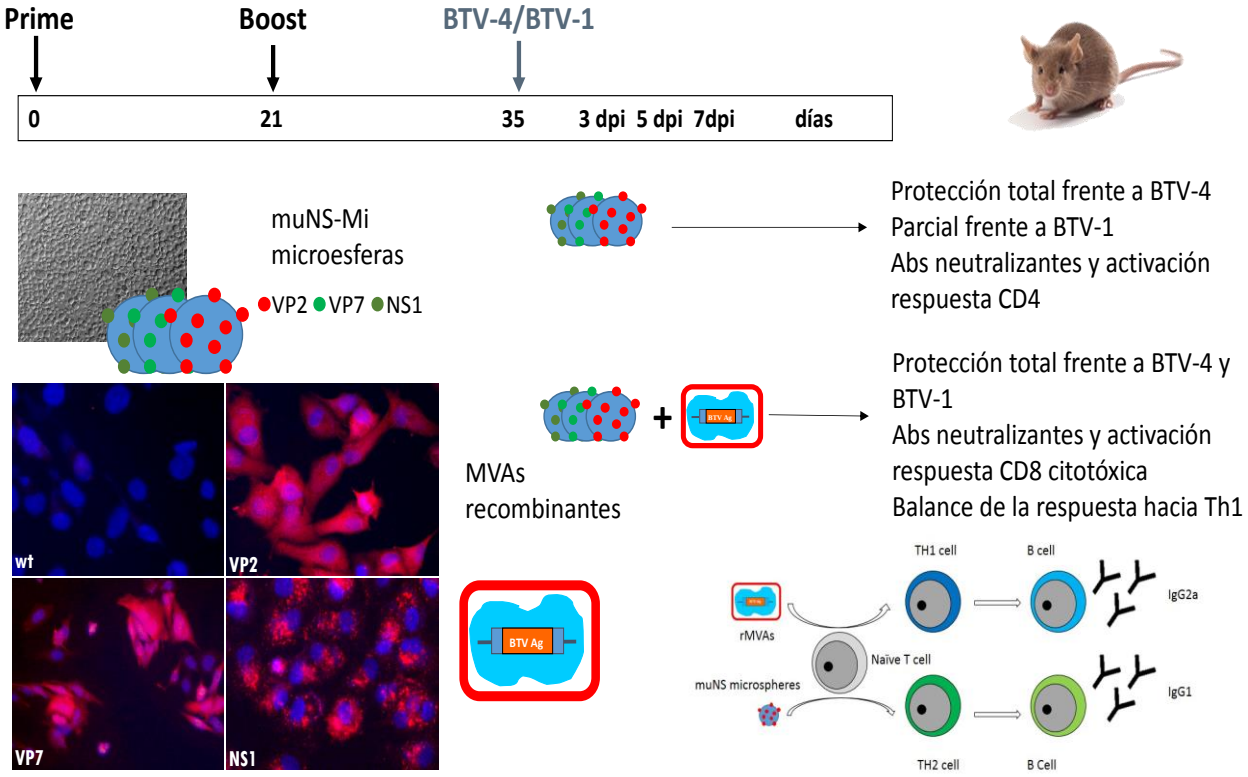
Desarrollo de estrategias combinadas de vacunación multiserotipo frente a BTV basadas en microesferas de la proteína muNS-Mi de reovirus aviar y el vector viral MVA que expresen los antígenos VP2, VP7 y NS1.

La lengua azul es una enfermedad hemorrágica que afecta principalmente a rumiantes y cuya mejor medida de control es la vacunación. Las vacunas comercializadas en Europa se basan en virus inactivado y adyuvantado con sales de aluminio o detergentes. Se ha observado que los animales, tras recibir repetidas inoculaciones de este tipo de vacunas, desarrollan un síndrome de carácter neurológico y artrítico denominado *"ASIA"*. Se han descrito 27 serotipos diferentes para el virus que provoca esta enfermedad. En este estudio nos hemos centrado en desarrollar una vacuna segura, con reactividad cruzada entre serotipos, eficaz y con capacidad diagnóstica basada en los antígenos VP2, VP7 y NS1 de BTV-4 incorporados en microesferas protéicas constituidas por la unión de múltiples moléculas de la región muNS-Mi de la proteína muNS del reovirus aviar (ARV) (MS-VP2/MS-VP7/MS-NS1). La inmunización de ratones IFNAR(-/-) con estas construcciones en ausencia de adyuvantes y siguiendo una estrategia *prime-boost*, aumentó significativamente los niveles de anticuerpos neutralizantes específicos de BTV-4 así como la activación de la respuesta celular T, preferentemente de tipo CD4+. Todos los animales inmunizados sobrevivieron al desafío homólogo con una dosis letal de BTV-4, y además se observó una protección parcial en los animales inmunizados que fueron infectados con una dosis letal de BTV-1, un serotipo filogenéticamente alejado de BTV-4.

Con el fin de mejorar la capacidad multiserotipo que ofrece la vacunación con las microesferas generadas en este trabajo se apostó por una estrategia combinada *prime-boost* de microesferas y rMVAs que expresaran los mismos antígenos de BTV-4, debido a las ventajas que ofrecen las vacunaciones heterólogas que incorporan vectores virales a la hora de aumentar la respuesta inmune celular T citotóxica, altamente involucrada el conferir inmunidad frente a múltiples serotipos de BTV. Así, ratones IFNAR(-/-) fueron inmunizados siguiendo esta estrategia *prime-boost* de vacunación heteróloga y en ausencia de adyuvantes, alcanzando niveles de anticuerpos neutralizantes similares a los conseguidos en la inmunización con microesferas y protegiendo a todos los animales frente a la infección homóloga con BTV-4. La combinación de las microesferas con los MVAs recombinantes, especialmente con rMVA-NS1, activó considerablemente la respuesta celular T de tipo CD8+ citotóxica y fue la responsable de conferir una protección total frente al desafío heterólogo con BTV-1 en ausencia de anticuerpos neutralizantes frente a este serotipo de BTV. Además,

se observaron diferencias en la relación IgG2a/IgG1 en el suero de los animales inmunizados solamente con microesferas en comparación con los inmunizados con la estrategia combinada, siendo la relación más alta en el suero de éstos últimos, lo que confirma que los rMVAs utilizados en la vacunación polarizan la respuesta hacia un patrón Th1, más relacionado con la activación de la respuesta celular.

Como conclusión, podemos afirmar que la combinación de las microesferas basadas en la proteína muNS del reovirus aviar con los MVAs recombinantes que expresan los mismos antígenos de BTV, (VP2, VP7 y NS1) es una estrategia vacunal prometedora para la inducción de protección frente a múltiples serotipos de BTV.



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VP2, VP7, and NS1 proteins of bluetongue virus targeted in avian reovirus muNS-Mi microspheres elicit a protective immune response in IFNAR(−/−) mice

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ABSTRACT

Vaccination is critical for controlling the spread of bluetongue virus (BTV). The inactivated BTV vaccines that are now being used in Europe are effective in preventing outbreaks of BTV but secondary effects associated to repetitive inoculation of aluminum-containing adjuvants and the need to develop safer, cross-reactive, and more efficacious vaccines with differential diagnostic capability have re-stimulated the interest in developing improved vaccination strategies against BTV. We have engineered a subunit BTV vaccine candidate based on proteins VP2, VP7, and NS1 of BTV-4 incorporated into avian reovirus (ARV) muNS-Mi microspheres (MS-VP2/MS-VP7/MS-NS1). IFNAR(−/−) mice immunized with MS-VP2/MS-VP7/MS-NS1 without adjuvant generated significant levels of neutralizing antibodies specific to BTV-4. In addition, vaccination stimulated specific T cell responses, predominantly CD4+, against the virus. Immunized mice were fully protected against a homologous challenge with a lethal dose of BTV-4 and partially cross-protected against a heterologous challenge with a lethal dose of BTV-1. These results support MS-VP2/MS-VP7/MS-NS1 as a promising subunit vaccine candidate against multiple serotypes of BTV as well as the use of microspheres as an alternative delivery method with potent intrinsic adjuvant activity.

1. Introduction

Bluetongue (BT) is a non-contagious, insect-transmitted disease of wild and domestic ruminants characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema that is caused by the bluetongue virus (BTV) (Maclachlan et al., 2009, 2014; Verwoerd, 2004). BTV infection of ruminants occurs throughout much of the tropical and recently also the temperate climate regions of the world, coincident with the distribution of specific species of *Culicoides* biting midges that are the biological vectors of this arbovirus (Gibbs and Greiner, 1994; Savini et al., 2008). BTV, the prototype of the *Orbivirus* genus, is a pathogen of livestock that is common throughout the world including Europe, and that causes serious periodic outbreaks (Patel and Roy, 2014). The incursion of BTV-8 in Northern Europe in 2008 has re-stimulated the interest to develop improved vaccination strategies against BTV. In particular, safer, cross-reactive, more efficacious vaccines with differential diagnostic capability have been pursued by multiple BTV research groups and vaccine manufacturers (Calvo-Pinilla et al., 2014).

The inactivated BTV vaccines that are now being used in Europe are effective in preventing outbreaks of BTV but they are serotype specific, and raise a broad immune response to all of the virus structural proteins. It invalidates established serological assays (e.g. ELISA) for routine surveillance and import/export testing, since they cannot distinguish between infected and vaccinated animals. In addition, an autoimmune/autoinflammatory syndrome induced by adjuvants (ASIA syndrome) linked to the repetitive inoculation of aluminum-containing adjuvants has been described in sheep. The syndrome shows an acute phase that affects less than 0.5% of animals in a given herd, and appears 2–6 days after an adjuvant-containing inoculation. It is characterized by an acute neurological episode with low response to external stimuli and

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acute meningoencephalitis (Lujan et al., 2013). In Spain, with the reemergence of bluetongue in 2008, animals received inactivated vaccines against two strains of BTV (strains 1 and 8) in alum, allowing sheep to receive four inoculations in less than one month. The ASIA syndrome appeared shortly after across the country with severe consequences for the sheep industry (Lujan et al., 2013).

The safety of alum as a vaccine adjuvant is currently being questioned (Tomljenovic and Shaw, 2011). For this reason, the generation of protective subunits vaccines with new adjuvants could be an alternative to the inactivated vaccines. Although in most vaccine protocols adjuvants are essential to provide appropriate danger signals for successful induction of immune responses (Gallucci et al., 1999), particulate vaccines appear to contain intrinsic adjuvant activity and to induce immune responses without the need of other adjuvants (Allsopp et al., 1996; Plebanski et al., 1998). Vaccine development has progressed significantly and has moved from whole microorganisms to subunit vaccines that contain only their antigenic proteins. Subunit vaccines are often less immunogenic than whole pathogens but incorporation of antigens into biomaterials can achieve a desired vaccine response. Such biomaterials, typically particulates that have been classically used as drug delivery carriers, can combine the antigen of interest and the ligands that direct those antigens to antigen-presenting cells (APCs), such as B cells, macrophages and dendritic cells (DCs) (Demento et al., 2011).

In the livestock industry, cost is a major factor in vaccine choice. For these reasons, a stable multiepitope particulate material for immunization should be a good candidate for a vaccine against BTV. The IC-tagging methodology, a tagging and inclusion-targeting system based on the ARV muNS virosomes (Brandariz-Nunez et al., 2010a, b; Brandariz-Nunez et al., 2011), is a simple, versatile, and efficient method for immobilizing active proteins in muNS-Mi inclusions (Microspheres or MS) in baculovirus-infected cells, that are easily purified (Brandariz-Nunez et al., 2011). The MS can simultaneously recruit several tagged proteins (Brandariz-Nunez et al., 2010a) that can be used to generate complexes and create multiepitope particulate material for immunization purposes that could have potential advantages as vaccines: (i) MSs are particulate matter, and particulate immunogens are the best for stimulating both humoral and cellular immune responses (Roy, 1996); (ii) MS-derived immunogens are cheap and very stable; and (iii) they should be biologically safe, because organisms would be immunized with proteins and not with genetic material or viruses.

The genome of orbiviruses consist of ten linear double-stranded RNA genome segments (Seg-1 to Seg-10) encoding structural proteins VP1 to VP7 and non-structural proteins, NS1, NS2, NS3/NS3a, and NS4 (Belhouchet et al., 2011; Mertens et al., 1984; Ratinier et al., 2011; Roy, 2005, 2008). Immunological studies to date have given many clues about what BTV proteins are more important to induce protective host immune responses against the virus. It has been demonstrated that neutralizing antibody responses (Jeggo et al., 1984b) and cytotoxic T lymphocytes (CTL) have a main role in protective immunity against BTV (Jeggo and Wardley, 1982; Jeggo et al., 1984a). For this reason, the BTV antigens included in a vaccine composition should stimulate humoral and cellular immune responses. Epitope mapping studies revealed that the major virus neutralizing epitopes are located in the protein VP2 (Roy, 1992). BTV-specific CTL have been studied in sheep, showing VP2 and NS1 as major CTL targets although VP3, VP5 and VP7 were also recognized by CTLs (Andrew et al., 1995; Janardhana et al., 1999; Rojas et al., 2014, 2011). Different strategies have been followed over the last 3 decades to develop novel recombinant vaccines for BTV, ranging from baculovirus expressed sub-unit vaccines to live virus vector vaccines Recently, our laboratory has showed that a heterologous prime boost vaccination strategy with DNA and the highly attenuated poxvirus vector modified vaccinia virus Ankara (rMVA) expressing VP2, VP7, and NS1 proteins of BTV-4, generated significant levels of neutralizing antibodies against BTV-4 in immunized IFNAR(−/−) mice. Furthermore, vaccination stimulated specific CD8+ T cell responses against these three BTV proteins. Importantly, the vaccine combination expressing NS1, VP2 and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4 infection and induced cross-protection against lethal doses of heterologous BTV-8 and BTV-1, two serotypes that are not related phylogenetically, suggesting that VP2, VP7, NS1 BTV proteins are key to the composition of a marker multiserotype vaccine against BTV (Calvo-Pinilla et al., 2012; Ortego et al., 2014).

In this study, we describe the generation of subunit BTV vaccines based on microspheres of ARV muNS-Mi containing BTV-4 VP2, VP7, and NS1 proteins. We show that immunization of mice deficient in type I IFN receptor (IFNAR(−/−)) with these microspheres and without adjuvants, achieves protective immunity and total or partial protection against homologous and heterologous infection with BTV-4 and BTV-1, respectively. These results indicate that the delivery of proteins VP2, VP7, and NS1 of BTV-4 in microspheres of ARV muNS-Mi protein is a promising subunit vaccine strategy to efficiently protect against BTV infection avoiding the use of added adjuvants.

2. Materials and methods

2.1. Virus and cells

Baby hamster kidney (BHK-21) (ATCC, Cat. No. CCL-10), chicken embryo fibroblast (DF-1) (ATCC, Cat. No. CRL-12203), and Vero (ATCC, Cat. No. CCL-81) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% fetal calf serum (FCS). Insect cells High Five (Invitrogen) and *Spodoptera Frugiperda* Sf9 (ATCC Cat. No. CRL-1711) were grown in TC-100 medium supplemented with 10% FCS. BTV serotype 4 (SPA2004/01) (BTV-4) and serotype 1 (ALG2006/01) (BTV-1) was used in the experiments. Standard virus titrations were performed in Vero cells. Virus stocks were generated by infection of confluent Vero cells using a multiplicity of infection (MOI) of 1. At 48 h post-infection (h.p.i.), or when total cytopathic effect (CPE) was visible, the cells and supernatants were harvested and centrifuged. The virus were released from the cells by three freeze and thaw cycles.

2.2. Mice

IFN $\alpha/\beta R^{o/o}$ IFNAR(−/−) 129/Sv mice and wild type 129/Sv mice were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout the study. Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal, INIA, Madrid, for 1 week before use in our experiments. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the Centro de Investigación en Sanidad Animal of the Instituto Nacional de Investigaciones Agrarias (Permit number: CEEA 2010-034). All efforts were made to minimize suffering.

2.3. Construction of recombinant baculoviruses for expressing untagged and IC-tagged VP2, VP7, and NS1 of BTV-4

All these recombinant baculoviruses were generated using the Bac-to-Bac system (Invitrogen, Barcelona, Spain) following the supplier protocols. The construction of Bac-muNS-Mi and Bac-GFP-muNS-Mi has been described previously (Brandariz-Nunez

Table 1
Primers used to generate recombinant baculoviruses for expressing IC-tagged VP2, VP7, and NS1 of BTV-4.

Primer	Sequence	Restriction site
Bac-VP2 F	GCG GAA TTC ATC ATG GAG GAG TTT GTC ATT C	EcoRI
Bac-VP2 R	GCA TAA GAA T GC GG CC GC CTA AAC GTT GAG TAA TTT CGT C	NotI
Bac-VP2-IC F	GCG GAA TTC ATC ATG GAG GAG TTT GTC ATT C	EcoRI
Bac-VP2-IC R	GCA TAA GAA T GC GG CC GC AAC GTT GAG TAA TTT CGT C	NotI
Bac-VP7 F	GCG GAA TTC ATC ATG GAC ACT ATC GTC GC	EcoRI
Bac-VP7 R	GCA TAA GAA T GC GG CC GC CTA CAC ATA GGC GGC GCG	NotI
Bac-VP7-IC F	GCG GAA TTC ATC ATG GAC ACT ATC GTC GC	EcoRI
Bac-VP7-IC R	GCA TAA GAA T GC GG CC GC CAC ATA GGC GGC GCG	NotI
Bac-NS1 F	GCG GAA TTC ATC ATG GAG CGC TTT TTG AG	EcoRI
Bac-NS1 R	GCA TAA GAA T GC GG CC GC CTA ATA CTC CAT CCA CAT CTG	NotI
Bac-NS1-IC F	GCG GAA TTC ATC ATG GAG CGC TTT TTG AG	EcoRI
Bac-NS1-IC R	GCA TAA GAA T GC GG CC GC ATA CTC CAT CCA CAT CT	NotI

Restriction sites are underlined.

et al., 2010b). Plasmids pcDNA3-VP2, pcDNA3-VP7 and pcDNA3-NS1 containing the sequences coding for BTV serotype 4 proteins VP2, VP7 and NS1 respectively have been previously described (Calvo-Pinilla et al., 2012, 2009). The sequences coding for the BTV proteins: VP2, VP7 and NS1 were PCR-amplified with specific primers (Table 1) from the previously mentioned plasmids. The PCR products were then cloned into plasmid pFastBac1 (Invitrogen) to obtain the transfer vectors that were used to generate the recombinant baculoviruses that express the three mentioned BTV proteins in insect cells, that were named: Bac-VP2, Bac-VP7 and Bac-NS1. To generate the recombinant baculoviruses that express the BTV proteins tagged with IC-Tag at their C-terminus (Bac-VP2-IC, Bac-VP7-IC and Bac-NS1-IC), the plasmid pFastBac1-IC (Brandariz-Nunez et al., 2010b) was used instead of pFastBac1.

2.4. Production and purification of muNS-Mi microspheres

The purification of muNS-Mi microspheres (MS) was previously described (3). In the present study we used a modification of the described protocol. Briefly, *S. Frugiperda* Sf9 cells cultured in suspension at a density of 1.5×10^6 cells/ml, were infected with 0.5 pfu/cell of the correspondent baculovirus. The infection was allowed to proceed for 6 or 7 days at 27 °C, and then the cells were centrifuged for 7 min at 1500×*g*. The pellet was washed once with PBS and then resuspended in 1/10 volume of RB buffer (10 mM Hepes pH 7.9; 10 mM KCl) containing 0.5% Triton X-100 and protease inhibitor cocktail. The lysate was sonicated to break nuclei and then centrifuged five minutes at 500×*g*. The pelleted microspheres were washed 4 times in the same buffer, once in RB buffer without Triton, and finally resuspended in a small volume of buffer RB. For protein quantification and PAGE analysis, the microspheres were previously dismantled in 10% SDS.

2.5. Immunofluorescence microscopy

For indirect immunofluorescence microscopy, Sf9 cell monolayers grown on coverslips were infected with the recombinant baculoviruses indicated for any particular experiment and incubated at 27 °C for 72 h. Then, the monolayers were washed twice with PBS and fixed for 15 min with 4% paraformaldehyde in PBS. Paraformaldehyde-fixed cells were washed twice with PBS, incubated for 4 min in permeabilizing buffer (0.5% Triton X-100 in PBS), and then blocked in PBS containing 2% bovine serum albumin for 1 h at room temperature. Then, the cells were incubated for 1 h at room temperature with antibodies raised against BTV 4 diluted in blocking buffer. After three washes with PBS, the cells were incubated for 30 min with secondary antibodies (Alexa 594 conjugated antibody against mouse IgG from Sigma–Aldrich) and DAPI. Coverslips were then washed six times with PBS and mounted on glass slides. Images were obtained with an Olympus DP-71 digital camera mounted on an Olympus BX51 fluorescence microscope. Images were processed with Adobe Photoshop (Adobe Systems, California, USA).

2.6. Prime-boost immunization and challenge with BTV in IFNAR(−/−) mice

Groups of five IFNAR(−/−) mice were immunized by homologous prime-boost vaccination with VP2/VP7/NS1, MS-VP2/MS-VP7/MS-NS1 or MS (non-immunized mice), administered 2 weeks apart. A suspension of 50 μg of each recombinant BTV-4 protein (VP2, VP7, and NS1) or 50 μg of each MS (MS-VP2, MS-VP7, and MS-NS1) was inoculated intraperitoneally. Two weeks after immunization all mice were subcutaneously inoculated with 5×10^2 PFUs of BTV-4 or 10^2 PFUs of BTV-1. Mice were bled before each immunization and virus challenge. Sera were tested for BTV-4 neutralizing antibodies by standard Virus Neutralization Test (VNT).

2.7. Detection of BTV-4

Whole blood was collected in EDTA from all animals at regular intervals after inoculation. The viruses were released from whole blood by three freeze/thaw cycles. The amount of infectious virus was measured by plaque assay on Vero cells.

2.8. BTV-4 neutralizing antibody detection in immunized mice by VNT

The VNT was used to determine neutralizing antibody titers against BTV-4. For plaque reduction assays, 2-fold dilutions of sera were mixed with 100 PFU of BTV-4, incubated for 1 h at 37 °C and then plated into monolayers of Vero cells. After 1 h, agar overlays were added and the plates were incubated for 5 days. The titer was determined as the highest dilution that reduced the number of plaques by 50%.

2.9. IFN-γ ELISPOT assays

ELISPOT assays were performed with Mouse IFN gamma ELISPOT Ready-SET-Go (eBioscience), according to the method recommended by the manufacturer. A total of 5×10^5 splenocytes were added to the well and stimulated with 10 μg of recombinant MS, MS-VP2, MS-VP7, or MS/NS1 proteins. Plates were incubated at 37 °C and 5% CO_2 for 18–20 h. As a positive control, PHA was used. Plates were scanned on an ImmunoSpot reader (Cellular Technology

Ltd.). Specific spots were counted using the Immuno-Spot software. The threshold value to consider a positive response by ELISPOT was that the number of specific spots/well had to be at least 2 times the average values found in negative control wells of each group, and that after subtraction of background values (MS protein stimulated splenocytes).

2.10. Detection of epitope-specific CD4+ and CD8+ T-cell responses by intra-cellular cytokine staining (ICCS)

Immunized 129 mice were sacrificed at 14 days post-booster and their spleens were harvested for analysis by ICCS assay. A total of 10^6 splenocytes were stimulated with 15 μg of recombinant MS or MS-VP2/MS-VP7/MS/NS1 proteins or left untreated during 18 h in RPMI 1640 supplemented with 10% FCS and containing brefeldin A (5 μg/ml) to increase the accumulation of gamma interferon (IFN-γ) in the responding cells. After stimulation, cells were washed, stained for the surface markers, fixed, permeabilized and stained intracellularly using the appropriate fluorochromes. To analyze the adaptive immune responses, the following fluorochrome-conjugated antibodies were used: CD4-FITC, CD8-PerCP and IFNγ-PE. All antibodies were from eBioscience. Data were acquired by FACS analysis on a FACSCalibur (Becton Dickinson). Analyses of the data were performed using FlowJo software version X0.7 (Tree Star, Ashland, OR). The number of lymphocyte-gated events was 5×10^5.

3. Results

3.1. Expression of untagged and IC-tagged proteins VP2, VP7, and NS1 of BTV-4

To obtain microspheres loaded with the BTV-4 proteins, we first generated recombinant baculoviruses to direct the expression of VP2, VP7 and NS1 containing the IC tag at the C-terminus. In parallel, we generated recombinant baculoviruses to express the untagged versions of the same proteins for comparison. Protein extracts from Sf9 cells infected with all the recombinant baculoviruses were analyzed by SDS–PAGE. Protein bands with the apparent molecular weight corresponding to VP7 (38 kDa), NS1 (64 kDa) and VP2 (111 kDa) are clearly seen in the coomassie-stained gel shown in Fig. 1 (indicated by arrows). Bands corresponding to the IC-tagged versions of the same proteins are also evident in lanes 4, 6 and 8 respectively, where the presence of the IC-tag produces an increase of approximately 7 kD in the apparent molecular weight of each protein.

To detect if the expression of the untagged and IC-tagged recombinant proteins were generating any kind of intracellular aggregates, Sf9 cells infected with the corresponding recombinant baculoviruses were subjected to immunofluorescence analysis using mouse polyclonal antibodies raised against BTV-4. The pictures at the right of Fig. 1 show that most of the expressed recombinant proteins distribute diffusely throughout the cytoplasm, although some small aggregates can be seen in the NS1-IC and VP7-IC samples due to an intrinsic characteristic of these two BTV proteins (Calvo-Pinilla et al., 2012).

3.2. Relocation of the BTV proteins to muNS-Mi microspheres

According to the IC-tagging method, the IC-tagged proteins should relocate to muNS-derived inclusions when co-expressed with muNS-derived, inclusion-forming proteins. Thus, we infected Sf9 insect cells with a recombinant baculovirus that expresses GFP-muNS-Mi, a fluorescent version of ARV muNS protein that forms intracellular inclusions able to recruit IC-tagged proteins (4), and co-infect them individually with the baculoviruses expressing untagged or IC-tagged BTV-4 proteins VP2, VP7 and NS1. The co-infected cells were analyzed by immunofluorescence using mouse polyclonal antibodies raised against BTV-4. Lanes 4, 5 and 6 in Fig. 2 show that IC-tagged NS1, VP2 and VP7 respectively (red), collected into big cytosolic inclusions that correspond to the same structures formed by GFP-muNS-Mi (green). On the other hand, the untagged BTV proteins did not co-localize at all with the GFP-muNS-Mi inclusions, showing that all three BTV-4 proteins were recruited to muNS-inclusions specifically driven by the IC tag as expected.

3.3. Purification of muNS-Mi microspheres loaded with BTV-4 proteins VP2, VP7 and NS1

To use as immunogens, we decided to use purified inclusions formed by muNS-Mi that are very regular in size and shape. We modified slightly our previous published purification protocol to improve the reproducibility of the inclusion formation and purification (See Methods). Under the conditions used in the present study, muNS-Mi formed very regular spherical inclusions (microspheres, MS) with a size ranging between 1.5 to 4 μm that are easily purified. To produce MS loaded with the three BTV-4 proteins, we performed

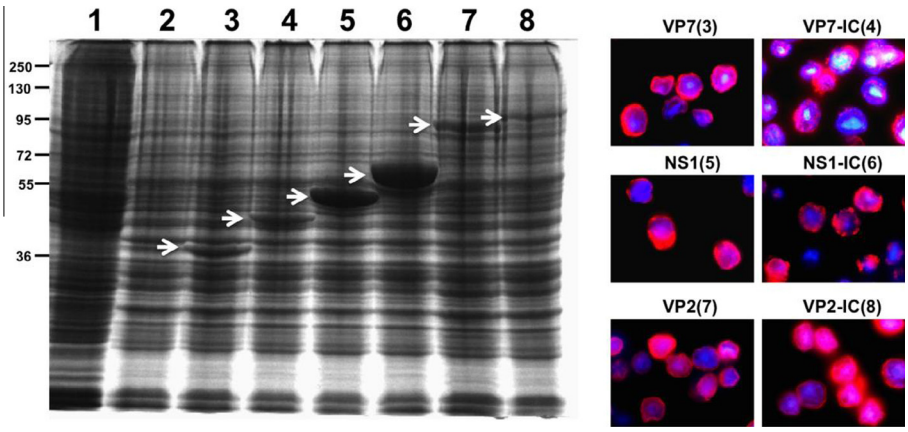

Fig. 1. Analysis of the baculovirus-driven expression of BTV-4 proteins in Sf9 cells. A Coomassie-stained gel is shown at the left of the figure, where protein extracts from insect Sf9 cells uninfected (lane 1), or infected with wild-type baculovirus (lane 2) are compared with those obtained with recombinant baculoviruses expressing the untagged BTV-4 proteins VP7, NS1 and VP2 (lanes 3, 5 and 7 respectively), and the same proteins bearing the IC-tag at their C terminus (lanes 4, 6 and 8). At the right side, the pictures show Sf9 cells where the intracellular distribution of the proteins indicated is detected by immunofluorescence using antibodies raised against BTV-4 (red). Nuclei were counterstained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

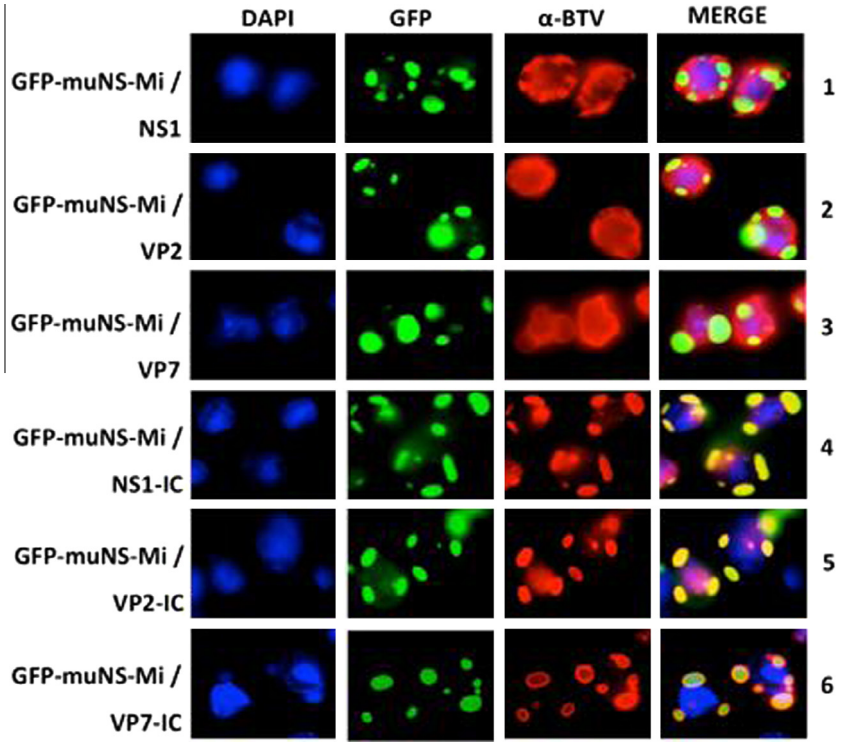

Fig. 2. Immunofluorescence analysis of the co-expression of ARV GFP-muNS-Mi and BTV-4 proteins. The pictures show Sf9 cells co-infected with the recombinant baculoviruses indicated at the left of each figure. The ARV-derived, GFP-muNS-Mi protein is shown in green (GFP), and the BTV-4 proteins were detected using polyclonal antibodies raised against BTV-4 (red). Nuclei were stained blue with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

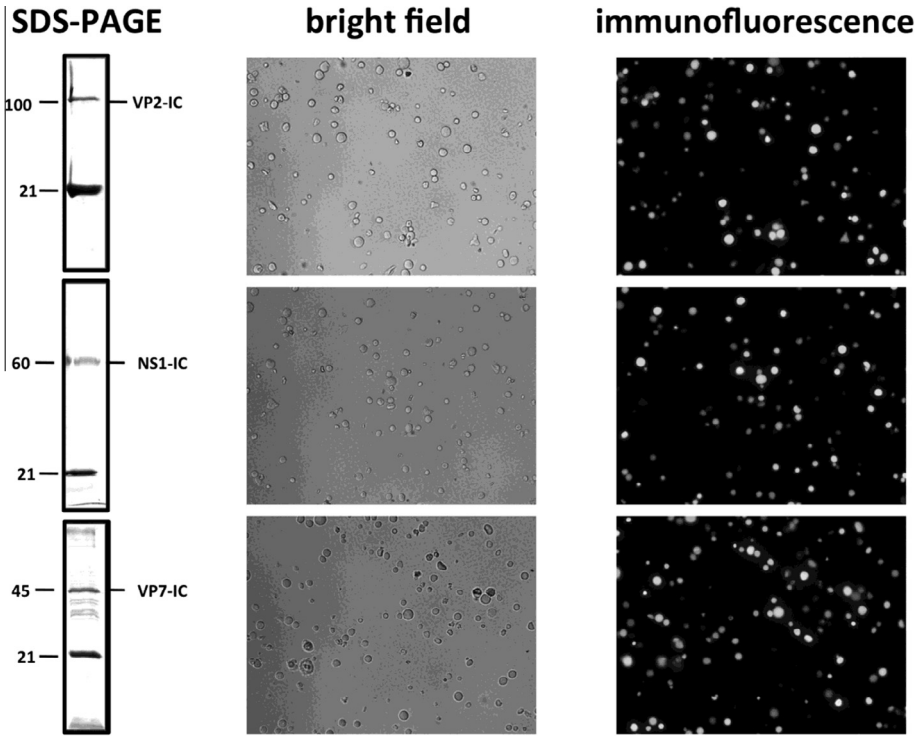

Fig. 3. Analysis of purified MS containing BTV-4 proteins. ARV muNS-Mi-derived microspheres containing IC-tagged BTV-4 VP2 (upper row), NS1 (middle row) or VP7 (lower row) were purified (see text) and analyzed by SDS–PAGE, bright field or immunofluorescence microscopy as indicated at the top of the figure. Positions corresponding to molecular weight markers are indicated at the left of the Coomassie-stained gels.

co-infections of Sf9 insect cells with the recombinant baculovirus expressing muNS-Mi on one hand, and the baculoviruses expressing each of the IC-tagged BTV-4 proteins on the other. The results of the purification are shown in Fig. 3. In all three cases, MS of the right size and appearance were purified (Fig. 3, middle column). The protein composition of each MS preparation was analyzed by

SDS–PAGE and coomassie staining (Fig. 3, left column). In the three MS preparations, a band corresponding to the correct IC-tagged BTV-4 protein was detected, together with a band of approximately 21 kD, corresponding to the avian reovirus muNS-Mi protein. The purified MS also reacted with antibodies against BTV-4 (Fig. 3, right column), while MS produced by muNS-Mi alone did not (not shown), further demonstrating the identity of the co-purified proteins.

3.4. Prime boost immunization with MS-VP2/MS-VP7/MS-NS1 protects IFNAR(−/−) mice against homologous BTV-4 infection

In order to characterize the efficacy as immunogens of MS-VP2, MS-VP7, and MS-NS1, adult IFNAR(−/−) mice were immunized twice two weeks apart with VP2,VP7, and NS1 recombinant BTV-4 proteins, with MS-VP2/MS-VP7/MS-NS1 or MS (non-immunized mice) by intraperitoneal injection. Two weeks after the second immunization, immunized and control mice were challenged subcutaneously with 5×10^2 PFUs of BTV-4. All non-immunized animals and mice immunized with recombinant VP2, VP7, and NS1 showed clinical signs and died. In contrast, 100% of the animals immunized with IC-tagged proteins did not show clinical signs throughout the experiment and they were completely protected against lethal challenge (Fig. 4A). The statistical analysis of the data using the Logrank test indicated significant differences ($p < 0.05$) between mice immunized with MS-VP2/MS-VP7/MS-NS1 and VP2/VP7/NS1 immunized or non immunized groups. The titers of infectious virus recovered in the blood after challenge with BTV-4 were determined in IFNAR(−/−) mice immunized with VP2, VP7, and NS1, or MS-VP2/MS-VP7/MS-NS1, and in non-immunized mice by plaque assay. Viremia was not detected in the MS-VP2/MS-VP7/MS-NS1 immunized mice; however, titers up to 2.8×10^2 PFU/ml were observed at 4 days post-challenge in non-immunized animals and up to 3.2×10^2 PFU/ml at day 5 post-challenge in VP2, VP7, and NS1 immunized mice (Fig. 4B). Furthermore, the presence of the BTV genome in blood of MS-VP2/MS-VP7/MS-NS1 immunized mice challenged with BTV-4 was analyzed by RT-qPCR (Toussaint et al., 2007) and all the animals were negative ($Ct \geqslant 38$) at all the analyzed days post-challenge (data not shown). These data indicate that the immunization of IFNAR(−/−) mice with a homologous prime boost with BTV-4 recombinant proteins VP2, VP7, and NS1 does not protect against a challenge with a lethal dose of BTV-4. In contrast, the same three BTV-4 proteins bound to microspheres of ARV muNS-Mi protein confer complete protection against BTV-4 after prime boost immunization.

3.5. Prime boost immunization with MS-VP2/MS-VP7/MS-NS1 elicits neutralizing antibodies against BTV-4 in IFNAR(−/−) mice

Neutralizing antibodies are critical to control BTV infection. Thus, to study the ability of VP2/VP7/NS1 and MS-VP2/MS-VP7/MS-NS1 to elicit humoral immune responses against BTV, we analyzed the levels of neutralizing antibodies present in the sera of IFNAR(−/−) mice immunized with two doses of these immunogens. The presence of neutralizing antibodies against BTV-4 in the sera of immunized and non-immunized mice was analyzed by virus neutralization tests. High titers of BTV-4 neutralizing antibodies were observed in immunized mice 2 weeks after booster treatment with MS-VP2/MS-VP7/MS-NS1 with a Log VNT^{50} of 1.84 ± 0.14 (Fig. 5). In contrast, the titer of neutralizing antibodies in sera of VP2/VP7/NS1 immunized mice was very low (0.9 ± 0.15). In addition, neutralization activity against BTV-4 was not detected in the serum of non-immunized mice ($VNT^{50} \leqslant 0.5$) at the analyzed time. This observation revealed that the inclusion of VP2, VP7, and NS1 in ARV muNS-Mi microspheres increases the capacity of these BTV antigens to induce neutralizing antibodies in immunized mice.

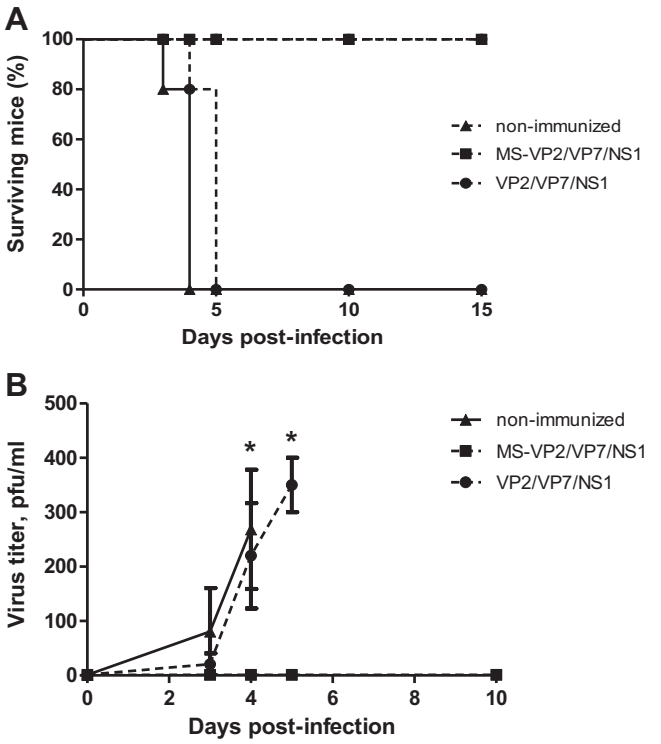

Fig. 4. Protection of MS-VP2/MS-VP7/MS-NS1 vaccinated IFNAR(−/−) mice against a lethal challenge BTV-4 challenge. Mice (8 weeks old, 5 per group) were immunized twice by homologous prime boost vaccination with recombinant VP2/VP7/NS1 soluble proteins or MS-VP2/MS-VP7/MS-NS1. Two weeks after immunization all mice were subcutaneously inoculated with 5×10^2 PFUs of BTV-4 (lethal dose). (A) Survival rates of immunized and non-immunized IFNAR(−/−) mice after inoculation with BTV-4. The mice were observed every 24 h for 15 days. Statistical differences between survival curves were calculated using the Logrank test. (B) Titers of BTV-4 recovered in blood of immunized and non-immunized IFNAR(−/−) mice after challenge. Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars. Asterisks indicate statistical significance calculated using the Student's *t* test ($p < 0.05$).

3.6. MS-VP2/MS-VP7/MS-NS1 immunization induces strong T cell immune response

To further analyze the protective immune response elicited by the MS-VP2/MS-VP7/MS-NS1 vaccines, the amount of IFN-γ-producing spleen cells after the immunizations was determined by ELISPOT. IFNAR(−/−) mice were immunized by homologous prime-boost vaccination with MS-VP2/MS-VP7/MS-NS1 or MS (control), administered 2 weeks apart. Two weeks after second immunization spleens were harvested and the splenocytes were stimulated with MS, MS-VP2, MS-VP7, or MS-NS1 in ELISPOT plates. As shown in Fig. 6, immunized mice developed detectable specific IFN-γ producing cells after stimulation with MS-VP2, MS-VP7, or MS-NS1 when compared to the non-immunized group. The increase in the level of IFN-γ producing cells after stimulation with MS-VP7, or MS-NS1 was significant when was analyzed by Student's *t*-test compared with the non immunized mice. Stimulation with MS-VP2 increased the level of IFN-γ producing cells although the difference was not significant when was analyzed by Student's *t*-test. In addition, the highest level of IFN-γ producing cells was observed in splenocytes of immunized mice that were stimulated with MS-VP7.

Immunization of IFNAR(−/−) mice with MS-VP2/MS-VP7/MS-NS1 induces a strong T cellular immune response as was observed by ELISPOT. In order to analyze the phenotype of the BTV-specific IFN-γ producing cells after immunization, we decide to immunize

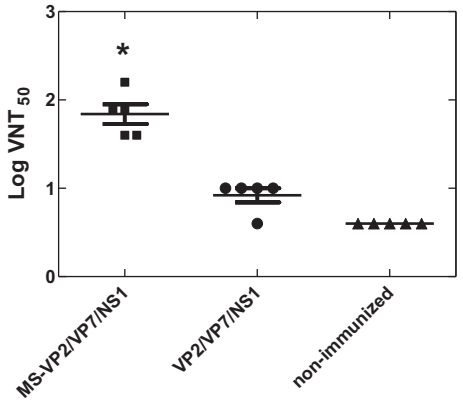

Fig. 5. Humoral immune response observed in IFNAR(−/−) mice vaccinated with VP2/VP7/NS1 or MS-VP2/MS-VP7/MS-NS1. Neutralizing antibodies specific of BTV-4 were analyzed in sera of immunized mice by VNT. Neutralization titers at day 15 post-booster treatment in sera of animals immunized with VP2/VP7/NS1 or MS-VP2/MS-VP7/MS-NS1 are shown. Non-Immunized, (▲) VP2/VP7/NS1 immunized, (●) and MS-VP2/MS-VP7/MS-NS1 immunized (■). Means are presented as bars (–). Asterisks (*) indicate statistically significant differences ($p < 0.05$) between immunized and non-immunized mice, calculated by signed rank test.

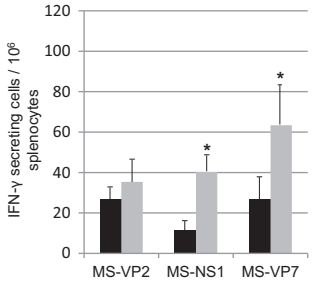

Fig. 6. ELISPOT assays measuring IFN-γ-secreting T cells in the spleen of immunized IFNAR(−/−) mice. Mice were immunized with MS-VP2/MS-VP7/MS-NS1 or MS as described in Materials and Methods. Splenocytes were harvested at day 14 post-boost. Mice inoculated with MS were used as non-immunized controls. Black (non-immunized) and gray (immunized) bars represent the SFC mean number after subtraction of background values (MS protein stimulated splenocytes) ± standard deviation for the ELISPOT within each group. 15 μg of MS or 10 μg of each protein (MS-VP2/MS-VP7/MS-NS1) per well were used as stimulus in each experiment.

129/sv wild type mice. These mice will reproduce better the T cell immune responses induced in the natural host by the vaccine, animals where the innate immune response is intact.

T cell immune responses induced in 129/sv mice by MS-VP2/MS-VP7/MS-NS1 14 days after the boost were measured by an intracellular cytokine staining assay (ICS) after the stimulation of splenocytes with MS-VP2/MS-VP7/MS-NS1 for 24 h. Intracellular IFNγ production by CD8+ T and CD4+ T cells was then determined by flow cytometry upon treatment of the cells with the golgi inhibitor, brefeldin A. MS-VP2/MS-VP7/MS-NS1 induced the expression of IFNγ by CD4+ and CD8+ T cells upon re-stimulation in immunized mice (Fig. 7) although the induction of CD4+ was significantly higher than the induction of CD8+ T cells. These data suggest that the strategy of immunization assayed elicits an immune cellular response in the mice, favoring preferentially specific CD4+ T cell responses.

3.7. MS-VP2/MS-VP7/MS-NS1 immunization partially protects IFNAR(−/−) mice against heterologous BTV-1 infection

In order to evaluate whether the subunit vaccine based on MS-VP2/MS-VP7/MS-NS1 from BTV-4 also confers protection against

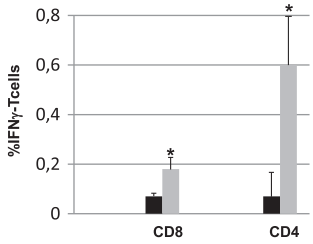

Fig. 7. Intracellular staining of IFN-γ, in T CD4+ and CD8+ cells of MS-VP2/MS-VP7/MS-NS1 immunized 129/sv mice. Two weeks after the second immunization, spleens were harvested and the splenocytes were stimulated with 15 μg of MS or MS-VP2/MS-VP7/MS-NS1 per well. At 24 h post-stimulation, intracellular IFN-γ production was analyzed in CD4-positive and CD8-positive cells by flow cytometry. Gray bars: mice immunized with MS-VP2/MS-VP7/MS-NS1; black bars: mice immunized with MS. The results represent the average of 3 mice after subtraction of background values (MS protein stimulated splenocytes) ± SD. Asterisks represent significant difference between samples, calculated by Mann–Whitney non parametric test ($p \leqslant 0.05$).

heterologous BTV infection, vaccinated IFNAR(−/−) mice were challenged with a lethal dose of BTV-1. The presence of serotype-specific neutralizing antibodies against BTV-4 and BTV-1 in the sera of immunized mice was analyzed by virus neutralization tests before challenge. Neutralizing antibodies against BTV-4 were observed in immunized mice two weeks after booster treatment with a Log VNT^{50} of 1.70 ± 0.20. In contrast, neutralizing antibodies against heterologous BTV-1 were not observed in the serum of immunized or non-immunized mice ($VNT^{50} \leqslant 0.3$) at the analyzed time. Immunized and control IFNAR(−/−) mice were challenged subcutaneously with 10^2 PFUs of BTV-1. After the challenge with BTV-1, 60% of the animals survived to the infection and all the non-immunized mice died after challenge with BTV-1 at day 7 post-infection (Fig. 8A). The statistical analysis of the data using the Logrank test indicated significant differences ($p < 0.05$) between immunized with and non immunized groups. The titers of infectious virus recovered in the blood after challenge with BTV-1 were determined in immunized and non-immunized IFNAR(−/−) mice by plaque assay (Fig. 8B). Titers up to 6.8×10^2 PFU/ml and 4.2×10^2 PFU/ml were observed in non immunized and immunized BTV-1 infected mice at 5 and 7 days post-challenge, respectively, and viremia disappeared in the immunized survival animals later in the infection. Overall, these data show that vaccination with MS-VP2/MS-VP7/MS-NS1 from BTV-4 exerts protective heterotypic immunity against heterologous infection and partially protects the animals against a heterologous challenge with BTV-1.

4. Discussion

Vaccination has been an effective approach to control BTV spread. Chemically inactivated vaccines, commercially available, protect against BTV infection but they are serotype specific and do not allow to distinguish between infected and vaccinated animals. Consequently, there are currently many efforts to develop new types of vaccines with improved safety and efficacy for a broad range of BTV serotypes (Boone et al., 2007; Calvo-Pinilla et al., 2012, 2009; Celma et al., 2013; Franceschi et al., 2011; Jabbar et al., 2013; Noad and Roy, 2009; Perrin et al., 2007; Roy and Noad, 2009). Most of these efforts concentrate on the development of subunit vaccines.

The generation of 'biomaterial vaccines' that can function as vaccine building blocks is attractive because they offer control over the physical and chemical properties of the material and they allow the addition of antigen, adjuvant, and target molecules for immune recognition. Conventional adjuvants, such as aluminum, elicit strong humoral responses but biomaterial particulate

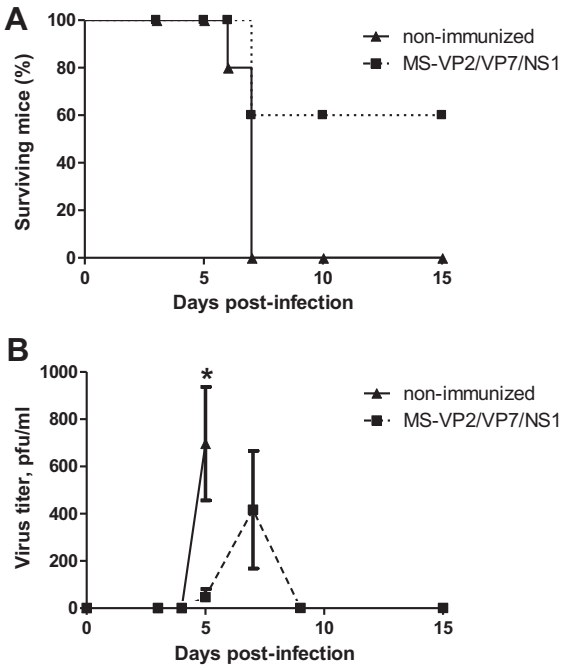

Fig. 8. Protection of MS-VP2/MS-VP7/MS-NS1 vaccinated IFNAR(−/−) mice against an BTV-1 challenge. Mice (8 weeks old, 5 per group) were immunized twice by homologous prime-boost vaccination with MS-VP2/MS-VP7/MS-NS1 (■) administered 3 weeks apart. Non-immunized mice were used as controls. Two weeks after immunization all mice were subcutaneously inoculated with 10^2 PFUs of BTV-1. (A) Survival rates of immunized and non-immunized IFNAR(−/−) mice after inoculation with BTV-1. The mice were observed every 24 h for 15 days. Statistical differences between survival curves were calculated using the Logrank test. (B) Titers of BTV-1 recovered in blood of immunized and non-immunized IFNAR(−/−) mice after challenge. Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars. Asterisks indicate statistical significance calculated using the Student's *t* test ($p < 0.05$).

systems offer the potential to produce humoral and cellular immune responses. Furthermore, the repetitive inoculation of aluminum-containing adjuvants can induce an autoimmune/autoinflammatory syndrome, a secondary effect of vaccination than can be avoided by using biomaterial vaccines. In this work, we have engineered a new and effective BTV subunit vaccine candidate based on microspheres of ARV muNS and BTV-4 VP2, VP7, and NS1 proteins. They are inherently safe and compatible with a DIVA approach.

Protein-based vaccines against BTV have been developed using single proteins or by combining various proteins in the same vaccine preparation. The most successful subunit vaccines against BTV are based in the co-expression of the four major structural proteins of the virus (VP2, VP5, VP3 and VP7), which results in their assembly into virus like particles (VLPs) (Roy, 1990; Roy et al., 1994, 1992). VLPs are particulate immunogens that present structural proteins in the correct conformation and give a good immune response but the production is expensive and in the livestock industry cost is a major factor in vaccine choice. Additionally, the expression of various proteins engaged in the formation of the VLPs must be precisely controlled in order to get the correct ratios between all the VLP components, and their purification presents a number of steps that are difficult to adapt to the industrial process. ARV microspheres, on the contrary, are very easily produced, purified, and very stable. When different serotypes of a particular virus are present, the microsphere method is easy and quickly adaptable by including MSs containing only those proteins that are different between serotypes. On the other hand, we have previously demonstrated the inclusion of different proteins together in the microspheres, and interaction between them to perform complex reactions (Brandariz-Nunez et al., 2010a, 2011). Thus, the microsphere method is fully compatible, when needed, with the formation of complex immunogenic epitopes by interaction between different viral proteins.

In a previous work, we developed a marker vaccine and a vaccination strategy against several BTV serotypes based on heterologous prime boost vaccination using DNA and rMVA expressing VP2, VP7, and NS1 proteins of BTV-4 (Calvo-Pinilla et al., 2012). IFNAR(−/−) mice inoculated with DNA/rMVA-VP2,-VP7-NS1 in a heterologous prime boost vaccination strategy generated significant levels of neutralizing antibodies against BTV-4 and stimulated specific CD8+ T cell responses against these three BTV proteins. Moreover, this vaccine combination expressing NS1, VP2 and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4. Due to the effectiveness of this vaccine composition in protection against BTV-4, VP2, VP7 and NS1 were chosen to generate particulate immunogens. The delivery of these three BTV antigens in microspheres of reovirus muNS-Mi protected the mice against a homologous challenge with BTV-4. Furthermore, mice immunized with MS-VP2/MS-VP7/MS-NS1 did not present viremia after challenge. In contrast, mice immunized with purified recombinant NS1, VP2 and VP7 proteins and without adjuvant were not protected against BTV-4 infection and infective virus was detected in blood. The efficiency at generating neutralizing antibodies has been described as a surrogate marker of protection in the BTV vaccines. Interestingly, MS-VP2/MS-VP7/MS-NS1 induced levels of neutralizing antibodies against BTV-4 similar to those mice immunized with DNA/rMVA-VP2,-VP7-NS1. In contrast, immunization of IFNAR(−/−) mice with purified recombinant NS1, VP2 and VP7 proteins without adjuvant induced low neutralization activity. Previous studies of immunization of IFNAR(−/−) mice with VP2 together with VP5 and VP7 of BTV-8 adjuvanted with Montanide ISA-50V showed induction of neutralizing antibody response (Calvo-Pinilla et al., 2014), albeit at lower levels than those observed when the antigens are delivered in microspheres. All these data confirm that the administration of VP2, VP7 and NS1 BTV-4 antigens in microspheres of avian reovirus muNS-Mi elicits a strong neutralizing activity and protection against BTV-4.

The study of the interaction of BTV with the immune system has showed that neutralizing antibodies (Jeggo et al., 1984b) and T cell immune response (Jeggo and Wardley, 1982; Takamatsu and Jeggo, 1989) have a role in protective immunity against BTV (Maclachlan et al., 2014). MS-VP2/MS-VP7/MS-NS1 are highly immunogenic in mice, inducing strong CD4+ T cell immune response, as it is observed in immunized wild type 129/sv mice. Typically, exogenous particulate antigens, once endocytosed, are loaded on major histocompatibility complex (MHC) class II molecules and presented to CD4+ T cells (Demento et al., 2011). Interestingly, MS-VP2/MS-VP7/MS-NS1 mainly elicited a CD4+ T cell response in the immunized mice although also a low CD8+ T cell response was also detected. The CD8+ T cell response has been reported as an alternative pathway in which particulate antigens might escape the endosome and the encapsulated antigen can be processed in the cytosolic space and be presented via MHC class I to CD8+ T cells (Demento et al., 2011).

The results showed that the prime boost vaccination with MS-VP2/MS-VP7/MS-NS1 conferred protection against a homologous challenge with BTV-4 in IFNAR(−/−) mice. Interestingly, this vaccination strategy also cross-protected partially against a heterologous challenge with BTV-1 in absence of neutralizing antibodies specific of this serotype. The importance of the inclusion of NS1 in the vaccine composition to induce cross-protection described in the IFNAR(−/−) mouse model (Calvo-Pinilla et al., 2012) has been also confirmed in cattle and sheep, natural host of BTV (Anderson et al., 2013; Rojas et al., 2014). The ability of including

non-structural proteins as NS1 is an additional advantage of ARV microspheres in comparison to VLPs, where only structural proteins can be incorporated. When the protein NS1 was included in the vaccine composition, in addition to VP2 and VP7 of BTV-4, the heterologous prime boost vaccination strategy DNA/rMVA elicited total cross-protection against lethal doses of heterologous BTV-8 and BTV-1 in absence of neutralizing antibodies specific of these two BTV serotypes. This vaccination stimulated strong specific CD8+ T cell responses against VP2, VP7, and NS1 proteins suggesting the importance of this cellular response in cross-protection (Calvo-Pinilla et al., 2012). Although the microspheres generated containing VP2, VP7, and NS1 mainly induce a CD4+ T cell response probably due to the size of the microsphere (diameter size between 1.5 and 4 μm), particles could be engineered to escape to endosomal and lysosomal compartments after APC internalization (Beaudette et al., 2009; Shen et al., 2006) and elicit a robust cell-mediated response. Previous work showed that solid inert beads with surface-adsorbed Ag stimulate CD8 T cell responses, with an optimal bead diameter size of 1 μm (Falo et al., 1995). However, other particulate immunogenic carriers such as virus-like particles (VLP) as well as many common viruses have a size range of 0.03–0.2 μm in diameter. In addition, in carboxylated polystyrene microspheres, the optimal carrier bead size was narrowly defined, ~0.04–0.05 μm, in the viral range. Moreover, Ag conjugated to this bead size was able to elicit combined humoral and CD8+ T cell immunity comparable to leading adjuvants for each arm of the immune response (Fifis et al., 2004). This previous studies suggest that the generation of new avian reovirus muNS-Mi microspheres with smaller diameter could elicit the CD8+ T cell response and improve the cross-protection against multiple serotypes of BTV.

In conclusion, in this work, we describe a novel BTV vaccine candidate based on microspheres of avian reovirus muNS and BTV-4 VP2, VP7, and NS1 proteins and show its resulting adaptive cellular immunogenicity, its ability to induce neutralizing antibodies specific of BTV-4, and its efficacy without adjuvants in protection against a homologous challenge with BTV-4. Furthermore, immunized mice are partially protected against a heterologous challenge against BTV-1. Although additional characterization in the BTV natural host will be necessary, data from the IFNAR(−/−) mice model suggest that VP2, VP7, and NS1 proteins of BTV-4 targeted in avian reovirus muNS-Mi microspheres could be a potential subunit multiserotype vaccine against BTV.

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Microspheres-prime/rMVA-boost vaccination enhances humoral and cellular immune response in IFNAR(−/−) mice conferring protection against serotypes 1 and 4 of bluetongue virus

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ABSTRACT

Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT), which affects domestic and wild ruminants. At the present, 27 different serotypes have been documented. Vaccination has been demonstrated as one of the most effective methods to avoid viral dissemination. To overcome the drawbacks associated with the use of inactivated and attenuated vaccines we engineered a new recombinant BTV vaccine candidate based on proteins VP2, VP7, and NS1 of BTV-4 that were incorporated into avian reovirus muNS-Mi microspheres (MS-VP2/VP7/NS1) and recombinant modified vaccinia virus Ankara (rMVA). The combination of these two antigen delivery systems in a heterologous prime-boost vaccination strategy generated significant levels of neutralizing antibodies in IFNAR(−/−) mice. Furthermore, this immunization strategy increased the ratio of IgG2a/IgG1 in sera, indicating an induction of a Th1 response, and elicited a CD8 T cell response. Immunized mice were protected against lethal challenges with the homologous serotype 4 and the heterologous serotype 1 of BTV. All these results support the strategy based on microspheres in combination with rMVAs as a promising multiserotype vaccine candidate against BTV.

1. Introduction

Bluetongue (BT) is a vector-borne viral disease of domestic and wild ruminants caused by Bluetongue virus (BTV), one of the most important livestock pathogens. (Saegerman et al., 2008). The disease is characterized by vascular injury that results in tissue necrosis, hemorrhage, and edema in ruminants (Maclachlan et al., 2009) and IFNAR(−/−) mice (Marin-Lopez et al., 2016). BTV belongs to the genus *Orbivirus* within the family *Reoviridae* with a non-enveloped virion and an icosahedral capsid. The genome is composed of double-stranded RNA (dsRNA) distributed in ten segments, encoding for seven structural proteins (VP1-VP7) and five/six nonstructural proteins (NS1, NS2, NS3/3A, NS4 and NS5) (Ratinier et al., 2011; Roy, 1992; Stewart et al., 2015). The virus is mainly transmitted between ruminant hosts through certain species of hematophagous *Culicoides* (*Diptera, Ceratopogonidae*) midges (Mellor et al., 2000). To date, 27 serotypes of BTV have been identified (Zientara et al., 2014) with two further putative/novel BTV serotypes identified so far (Maan et al., 2015). Since 1998 at least 8 serotypes have been detected within the European Union (Zientara and Sanchez-Vizcaino, 2013) and the introduction of new BTV serotypes is a permanent threat to the region. The constant arrival of new BTV serotypes re-emphasizes the importance of making multiserotype and more effective vaccines than those that are currently available. Although conventional vaccines have controlled or limited BTV spreading in the past, they cannot address the need for cross-protection among serotypes (Marín-López et al., 2016). Furthermore, modified live virus (MLVs) and inactivated vaccines currently used in Europe do not allow the differentiation of infected from vaccinated animals (DIVA).

In previous work in our laboratory, we demonstrated that the immunization of IFNAR(−/−) mice with an experimental subunit

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vaccine based on VP2, VP7, and NS1 proteins of BTV-4 incorporated into avian reovirus (ARV) muNS-Mi microspheres (MS-BTV) and without adjuvant protected against a homologous challenge with a lethal dose of BTV-4. Furthermore, the vaccine partially cross-protected against a heterologous challenge with a lethal dose of BTV-1 (Marin-Lopez et al., 2014). Immunized mice generated significant levels of neutralizing antibodies specific to BTV-4 and T cell responses, predominantly $CD4^{+}$. Cellular immune responses, specially cytotoxic T lymphocyte responses (CTL) have been demonstrated to be important in clearance of homologous and heterologous serotypes of BTV (Jeggo et al., 1985) and *in vitro* studies showed that BTV-specific ovine and murine CTL were cross-reactive among serotypes (Jones et al., 1996; Takamatsu and Jeggo, 1989). Furthermore, CD8 T cell epitopes have been identified in sheep and the murine model of infection from VP7 and NS1 proteins of BTV-8 (Rojas et al., 2011, 2014) and CTL epitopes have been described in sheep from VP2 and NS1 proteins of BTV-1 (Andrew et al., 1995; Janardhana et al., 1999).

In the present work, we developed a new vaccination strategy based on a heterologous prime-boost strategy with the particulate subunit vaccine MS-BTV and with the viral vaccine vector MVA expressing BTV antigens. Particulate immunogens are best for stimulating both humoral and cellular immune responses (Roy, 1996). Moreover, they are cheap, very stable, do not require the use adjuvants due to their intrinsic adjuvant effect, and are biologically safe. On the other hand, it has been described the use of rMVAs as a potent inductor of CD8 T cellular immune responses when used as a heterologous boost vaccination following a strong priming agent expressing the same antigen (Cottingham and Carroll, 2013; Whelan et al., 2009). In order to improve the serotype cross-protection of the experimental vaccine, we focused the vaccine composition on the VP2, VP7 and NS1 proteins of BTV-4. These proteins have been generally described to induce cross-serotype helper T-cell or cytotoxic T-cell responses.

2. Materials and methods

2.1. Virus and cells

Chicken embryo fibroblasts (DF-1) (ATCC, Cat. No. CRL-12203) and Vero (ATCC, Cat. No. CCL-81) cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. Insect cells High Five (Invitrogen) were grown in TC-100 medium supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. BTV serotype 4 (SPA2004/02) (BTV-4) isolated from whole sheep blood and serotype 1 (ALG2006/01) (BTV-1) isolated from sheep spleen were used in the experiments. BTV and MVA virus stocks and titrations were performed as previously described (Calvo-Pinilla et al., 2009).

2.2. Mice

IFN $\alpha/\beta R^{o/o}$ IFNAR(−/−) 129/Sv mice and wild type 129/Sv mice were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout. Upon reception, the mice were held for 7 days for acclimatization under pathogen-free conditions in the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal (INIA-CISA), Madrid. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the INIA-CISA and Comunidad de Madrid (Permit number: PROEX 037/15).

2.3. Generation of muNS-Mi microspheres (MS-VP2/VP7/NS1), and recombinant MVAs expressing VP2, VP7 and NS1 BTV-4 proteins (rMVA-VP2/VP7/NS1)

The production and purification of muNS-Mi-VP2/VP7/NS1 microspheres using the baculovirus expression system and the generation of rMVA-VP2/VP7/NS1 have been previously described (Brandariz-Nunez et al., 2010; Marin-Lopez and Ortego, 2016; Marin-Lopez et al., 2014).

2.4. In silico T CD8 epitope prediction

Amino acid sequences for the non-structural NS1 protein (NCBI accession number: AM778441.1) and structural VP2 protein (NCBI accession number: KP821068.1) of BTV-4 (SPA2004/02) were analized using three prediction algorithms available on the web: Immuno Epitope DataBase (IEDB Analysis Resource) (www.iedb.org), SYFPEITHI (www.syfpeithi.de), and BIMAS (www.bimas.cit.nih.gov/) for the H-2-Db MHC class I for 129/Sv mice to identify T CD8 epitopes that could be good binders to H-2-Db MHC. Theoretical T-cell epitopes were chosen by a combination of the best score in these databases (Table 1).

2.5. Peptides

The selected peptides (Table 1) were purchased from Proteogenix (Schiltigheim, France) and Sigma-Aldrich (The Woodlands, USA). Peptides were >95% pure (HPLC%). They were re-constituted in DMSO or Dimetilformamide according to the manufacturer instructions and kept frozen at −20 °C until use. All peptides were diluted in culture media: RPMI-1640 (Gibco, GreenIsland, NY) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) antibiotic-antimycotic solution (Gibco), 1% (v/v) non-essential aminoacids and 2 mM glutamine (Gibco).

2.6. Studies of cellular immune response in IFNAR(−/−) mice

Three groups of IFNAR(−/−) mice (n = 4) were immunized following a homologous prime-boost regimen with rMVA-VP2, rMVA-NS1 or MVA-wild type (non-immunized group) three weeks apart. All animals were sacrificed at 14 days post-booster and their spleens were harvested for analysis by ELISPOT and intra-cellular cytokine staining (ICCS) as previously described (de la Poza et al., 2015; Marin-Lopez et al., 2014).

2.7. Prime-boost immunization and challenge with BTV in IFNAR(−/−) mice

Groups of five IFNAR(−/−) mice were immunized by homologous prime-boost vaccination with MS-VP2/VP7/NS1 (50 μg of each

Table 1
Peptides selected from the epitope prediction in H-2 Db haplotype.

Protein	Position	Sequence	IEDB	SYFPEITHI	BYMAS
VP2	147	IYYDYFPL	0.3	–	–
	694	YLIQNSTGL	0.8	25	720.000
	731	LNVINFLPL	0.5	23	720.000
	743	VQDNISYW	0.8	–	–
	782	KSFYNFIRF	1.4	23	3120
	139	AKTANADTI	0.4	26	220.000
NS1	125	SALVNSERV	0.2	28	51.480
	152	GQIVNPTFI	0.2	28	720.000
	14	YANATRTFL	0.7	16	2.600
	222	IQLINFLRM	0.2	25	792.000

Using a combination of three epitope T prediction algorithms (IEDB, SYFPEITHI and BYMAS), peptides from VP2 and NS1 proteins of BTV-4 were selected and synthesized.

per mice) or by heterologous prime-boost vaccination with MS-VP2/VP7/NS1 (50 μg/mice) and rMVA-VP2/VP7/NS1 (10^7 PFU/mice), or prime-boosted with MS (50 μg/mice) and 3×10^7 PFU per mice of MVA-wt (non-immunized) administered by intraperitoneal injection three weeks apart. Two weeks after the second immunization all mice were subcutaneously inoculated with 5×10^2 PFUs of BTV-4 or 10^2 PFUs of BTV-1. Clinical data were recorded as described previously (de la Poza et al., 2013).

2.8. Detection of BTV-4 and BTV-1 in blood

Whole blood was collected in EDTA from all animals at regular intervals after viral challenge. The viruses were released from whole blood by three freeze/thaw cycles. The amount of infectious virus was measured by plaque assay on Vero cells.

2.9. BTV-4 and BTV-1 neutralizing antibody detection in immunized mice by viral neutralization test (VNT) and isotyping ELISA assay for antibodies specific of VP2 protein

The VNT was used to determine neutralizing antibody titers against BTV-4 prior infection. For plaque reduction assays, 2 fold dilutions of sera were mixed with 100 PFU of BTV-4, incubated for 1 h at 37 °C and then plated into monolayers of Vero cells. After 1 h, agar overlays were added and the plates were incubated for 5 days. The titer was determined as the highest dilution that reduced the number of plaques by 50%.

Isotype determination was performed by ELISA using a mouse immunoglobin isotyping kit (Biorad), using a fixed dilution of sera (1/50). As antigen in this assay, 96 well plate was coated with 150 ng/well of recombinant VP2 protein from BTV-4 expressed in Bac-To-Bac Baculovirus expression System, (Invitrogen) (Calvo-Pinilla et al., 2012).

3. Results

3.1. Heterologous prime boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 protects IFNAR(−/−) mice against homologous BTV-4

With the aim to analyze the efficacy of the combination of MS-VP2/VP7/NS1- rMVA-VP2/VP7/NS1 as immunogen to protect against BTV-4 infection, two groups of mice were immunized with MS-VP2/VP7/NS1. Three weeks apart, one group of mice was boosted with rMVA-VP2/VP7/NS1 and the other group received a second immunization with MS-VP2/VP7/NS1. A third group of mice

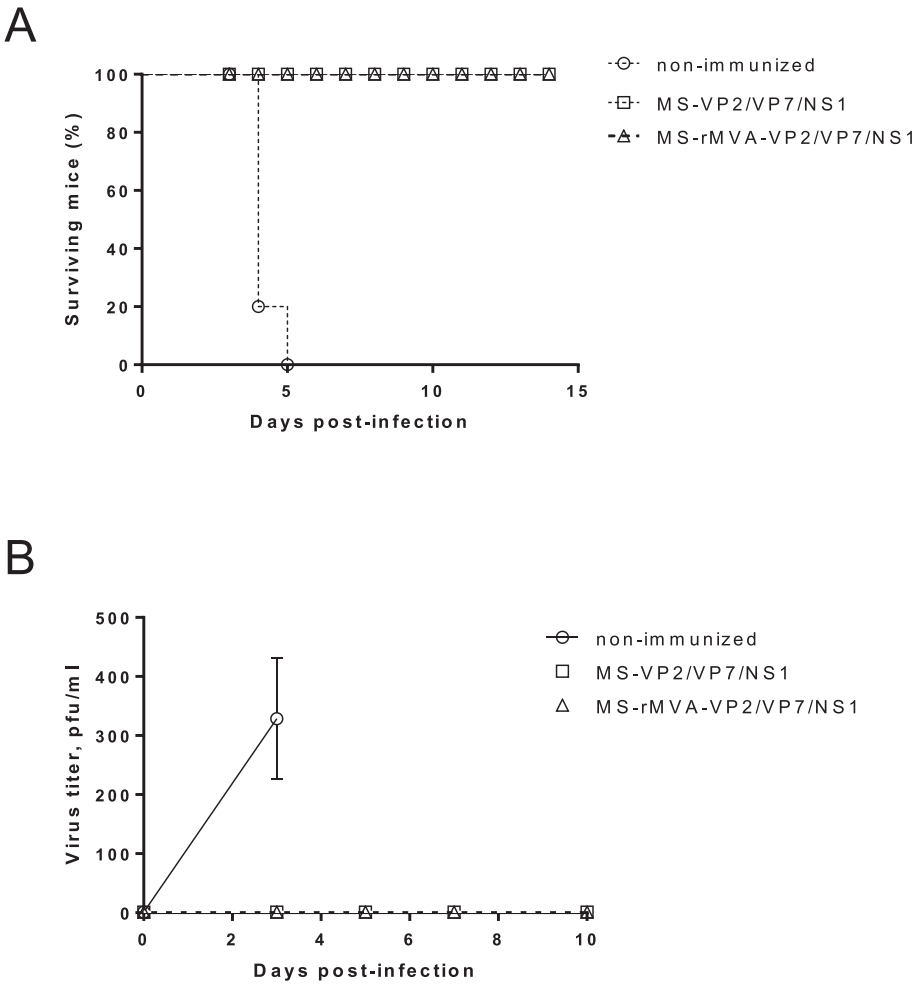

Fig. 1. Protection of MS-rMVA-VP2/VP7/NS1 vaccinated IFNAR(−/−) mice against a lethal challenge with BTV-4. Mice (8 weeks old, 5 per group) were immunized by prime-boost vaccination with MS-VP2/VP7/NS1 or heterologous MS-rMVA-VP2/VP7/NS1. Two weeks after immunization all mice were subcutaneously inoculated with 5×10^2 PFUs of BTV-4 (lethal dose). (A) Survival rates of immunized and non-immunized IFNAR(−/−) mice after inoculation with BTV-4. The mice were observed every 24 h for 15 days. (B) Titers of BTV-4 recovered in blood of immunized and non-immunized IFNAR(−/−) mice after challenge. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars.

was primed with MS and boosted with MVA-wt (non-immunized group). Two weeks after the second immunization, mice were challenged subcutaneously with 5×10^2 PFUs of BTV-4. All non-immunized animals showed clinical signs (the most severe signs, eye swelling, lethargy and hypothermia appeared at day 4) and died between days 4–5 post-infection. In contrast, 100% of the animals immunized with MS-VP2/VP7/NS1 or the combination of MS-VP2/VP7/NS1 with rMVA-VP2/VP7/NS1 did not show clinical signs throughout the experiment and they were completely protected against the lethal challenge with BTV-4 (Fig. 1A). The titers of infectious virus recovered in the blood after challenge with BTV-4 were determined in all animals by plaque assay and titers up to $3.3 \pm 1.0 \times 10^2$ PFU/ml were observed at day 3 post-challenge in non-immunized animals (Fig. 1B). Viremia was not detected in the immunized mice.

3.2. Prime boost immunization with MS-VP2/VP7/NS1 and MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 elicits neutralizing antibodies against BTV-4 in IFNAR(−/−) mice increasing the ratio IgG2a/IgG1

In order to analyze the humoral immune response elicited in mice immunized with the homologous and the heterologous strategies, virus neutralization test (VNT) and isotyping ELISA assay were performed using the sera of immunized and non-immunized animals collected 2 weeks after the booster treatment. Similar titers of BTV-4 neutralizing antibodies were observed in the sera with a VNT_{50} of 1.825 ± 0.14 and 1.9 in MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 and MS-VP2/VP7/NS1 immunized mice, respectively (Fig. 2A). Neutralization activity against BTV-4 was not detected in serum of non-immunized mice ($VNT_{50} \leq$ 0.6) at the analyzed time. This observation revealed that combining microspheres and recombinant MVAs expressing BTV antigens achieved similar high levels of neutralizing antibodies to those induced by microspheres alone.

Sera analyzed by ELISA showed an increase in the induction of IgG subtypes 1, 2a and 2b in the animals boosted with rMVA-VP2/VP7/NS1 compared with those immunized with two doses of MS-VP2/VP7/NS1 (Fig. 2B). Furthermore, the IgG2a/IgG1 ratio was clearly increased in the group boosted with MVA compared to the group immunized with two doses of microspheres (Fig. 2C). These data indicate that the introduction of recombinant MVAs in the vaccine composition improve the humoral response induced just by microspheres, enhancing the ratio of IgG2a/IgG1, suggesting an activation of a Th1-biased immune response.

3.3. MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 immunization elicits strong cellular immune response

To further analyze the cellular immune response elicited by the MS-VP2/VP7/NS1+ rMVA-VP2/VP7/NS1 vaccine, the amount of IFN-γ-producing spleen cells after the immunizations was determined by ELISPOT. IFNAR(−/−) mice were immunized by prime-boost vaccination with MS-VP2/VP7/NS1, MS-rMVA-VP2/VP7/NS1 or MS (control), administered three weeks apart. Two weeks after the second immunization spleens were harvested and the splenocytes were stimulated with MS, MS-VP2, MS-VP7, or MS-NS1 in ELISPOT plates. Animals immunized with the combined strategy significantly increased the level of IFN-γ producing cells after stimulation when compared to the group immunized with MS or the non-immunized group (Fig. 3).

3.4. Prediction and assessment of peptide binding from VP2 and NS1 protein to H-2-Db haplotype as T CD8 epitopes

MVA vaccines are expected to induce a strong $CD8^+$ T cell immune response. To identify epitopes triggering virus specific $CD8^+$

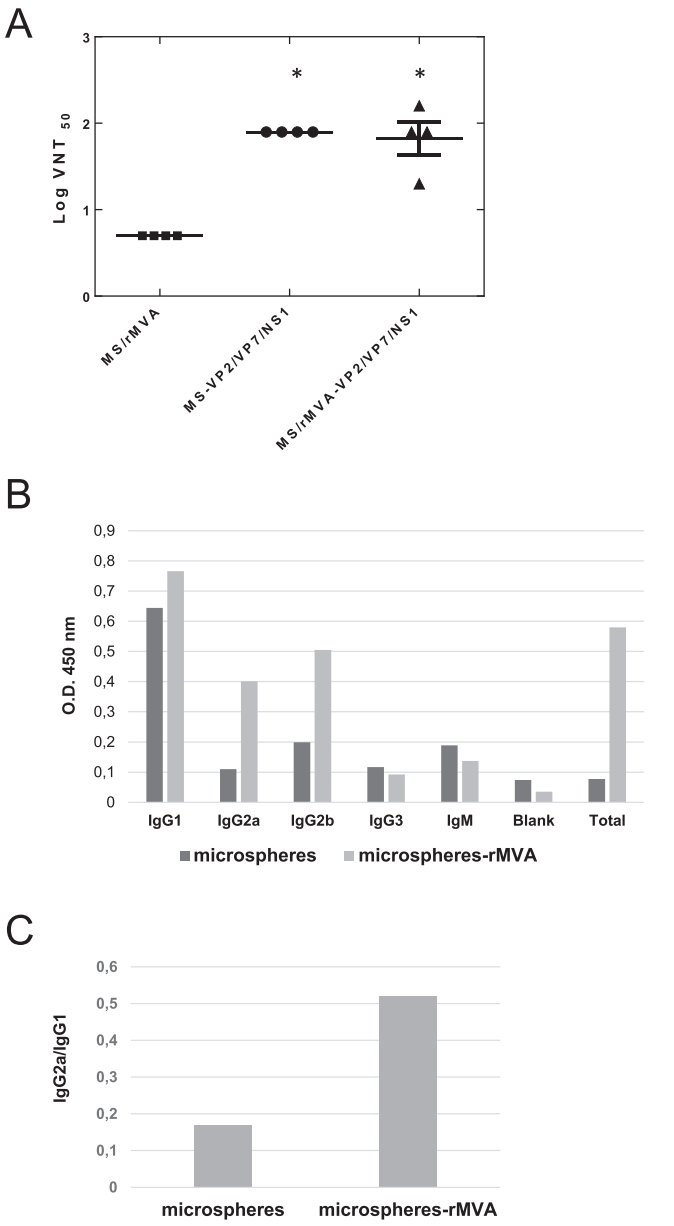

Fig. 2. Humoral response observed in IFNAR(−/−) mice vaccinated with **MS-VP2/VP7/NS1** or **MS-rMVA-VP2/VP7/NS1.** (A) Neutralizing antibodies specific of BTV-4 were analyzed in sera of immunized mice by VNT. Neutralization titers at day 15 post-boost treatment in sera of animals immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 are shown. Means are presented as bars and standard deviations are shown as error bars. Asterisks (*) indicate statistically significant differences ($P < 0.05$) between immunized and non-immunized mice, calculated by signed rank test. (B) IgG1 and IgG2a, IgG2b, IgG3, and IgM antibody levels were measured in 15 post-booster treatment pools of sera of animals immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 by ELISA. (C) IgG2a/IgG1 O.D. ratio.

T cell responses we used a combination of three epitope prediction algorithms and 6 peptides from VP2 and 4 peptides from NS1 proteins from BTV-4 were selected and synthetized (Table 1). The ability of these peptides to stimulate the proliferation of IFNγ positive $CD8^+$ T cells and to induce the expression of CD107a on the surface of this population/to bind H-2 Db and Kb molecules was assessed by ICCS assay in splenocytes from immunized animals with VP2 or NS1 proteins vectorized through recombinant MVAs. None of the six VP2 selected peptides induced significant levels of IFN-γ in $CD8^+$ T cells in the immunized animals after *in vitro*

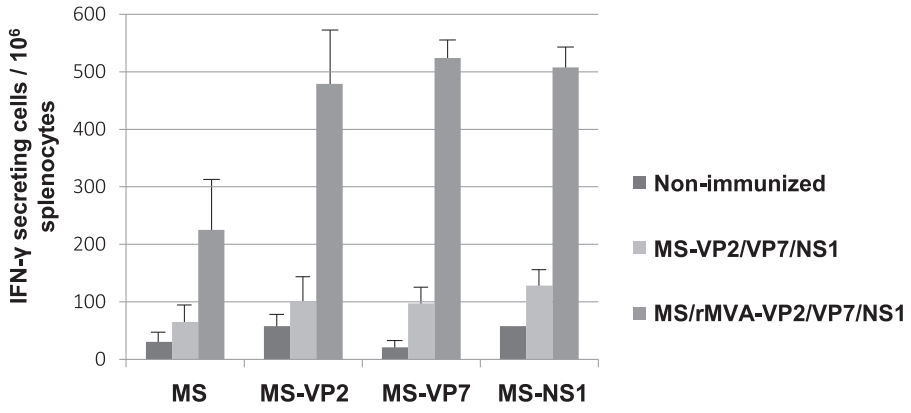

Fig. 3. ELISPOT assays measuring IFN-γ-secreting T cells in the spleen of immunized IFNAR(−/−) mice. Mice were immunized with MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 as described in Materials and Methods. Splenocytes were harvested at day 14 post-boost. Mice inoculated with MS were used as non-immunized controls. Bars represent the IFN-γ spot-forming cells (SFC) mean number ± standard deviation within each group. 10μg of each protein (MS, MS-VP2, MS-VP7, and MS-NS1) per well were used as stimulus in each experiment.

stimulation. (Fig. 4A). In contrast, one of the four NS1 selected peptides, NS1-152 induced a significant recall INFγ response in $CD8^+$ T cells from the immunized animals (Fig. 4B). These data confirm that NS1-152 peptide is a CD8 epitope in the 129 mouse strain. We next examined the ability of NS1-152 peptide to induce the expression of CD107a on the surface of IFN-γ positive $CD8^+$ T cells, as a surface marker of CTL degranulation. The NS1-152 peptide significantly induced the presence of CD107a on the surface of restimulated $CD8^+$ T cells indicating that NS1-152-specific $CD8^+$ T cells display CTL activity (Fig. 4C). These results suggest that NS1-152 is an immunodominant epitope for $CD8^+$ T cell responses in IFNAR(−/−) mice.

3.5. MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 immunization induces strong $CD8^+$ T cell immune response and promotes cytotoxic activity

To assess the phenotype of the BTV-specific IFNγ producing cells after *in vitro* restimulation observed by ELISPOT assay, we immunized mice with MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1. T cell immune responses were measured by ICCS after the stimulation of splenocytes with the NS1-152 peptide. Re-stimulation with the peptide induced high expression of IFNγ by $CD8^+$ T cells in the group of mice boosted with rMVA-VP2/VP7/NS1 (Fig. 5A). Furthermore, the level of $CD8^+$ T cells secreting CD107 augmented upon re-stimulation with NS1-152 peptide in this group of animals (Fig. 5B). These data suggest that the booster with rMVA-VP2/VP7/NS1 of animals primed with MS-VP2/VP7/NS1 elicits an specific $CD8^+$ T cell response, exhibiting marked cytotoxic activity.

3.6. Heterologous prime boost immunization with MS-VP2/VP7/NS1+rMVA-VP2/VP7/NS1 protects IFNAR(−/−) mice against heterologous BTV-1 infection

In order to analyze the efficacy of the combination of MS-VP2/VP7/NS1- rMVA-VP2/VP7/NS1 as a vaccine candidate against multiple serotypes of BTV, one group of animals was immunized following a prime-boost strategy, while a second group was primed with MS and boosted with MVA-wt (non-immunized mice). Both groups were challenged with a lethal dose of BTV-1. Non-immunized animals died between day 5 and 6 post-infection, showing the most severe clinical signs at days 4 and 5 and a peak of viraemia at day 5. In contrast, all immunized animals survived after challenge and they did not show clinical signs or viraemia after infection (Fig. 6). These results indicate that the heterologous prime-boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 reaches the levels of protection conferred by the homologous immunization against BTV-4 achieving a 100% of survival and also provides a total protection in absence of remarkable clinical signs and viraemia against the heterologous challenge with BTV-1.

4. Discussion

Current BT vaccines commercially available include both live attenuated and inactivated vaccines. They are effective but serotype specific and incompatible with serological assays to differentiate infected from vaccinated animals. In the last years, new experimental DIVA vaccines are being developed trying to generate broad cross-protection among BTV serotypes.

In previous work, we developed a subunit BTV vaccine candidate based on the proteins VP2, VP7, and NS1 of BTV-4 incorporated into avian reovirus muNS-Mi microspheres. IFNAR(−/−) mice immunized with MS-VP2/VP7/NS1 without adjuvant generated significant levels of neutralizing antibodies specific of BTV-4, a specific CD4 T cell response, and immunized mice were fully protected against an homologous challenge with a lethal dose of BTV-4 and partially protected against the heterologous BTV-1 (Marin-Lopez et al., 2014). In the present work, we combined this subunit vaccine with rMVAs expressing the same BTV antigens in a prime-boost regimen to improve the protection against heterologous serotypes of BTV. The results show that the prime boost immunization with MS-VP2/VP7/NS1 and rMVA-VP2/VP7/NS1 confers total protection against a homologous challenge with BTV-4 and heterologous with BTV-1 in IFNAR(−/−) mice. This new strategy elicits similar levels of neutralizing antibodies specific of BTV-4 that the strategy based only in microspheres. Interestingly, the boost with rMVA-VP2/VP7/NS1 increases IgG2a and IgG2b antibody levels in sera and enhances the ratio of IgG2a/IgG1,

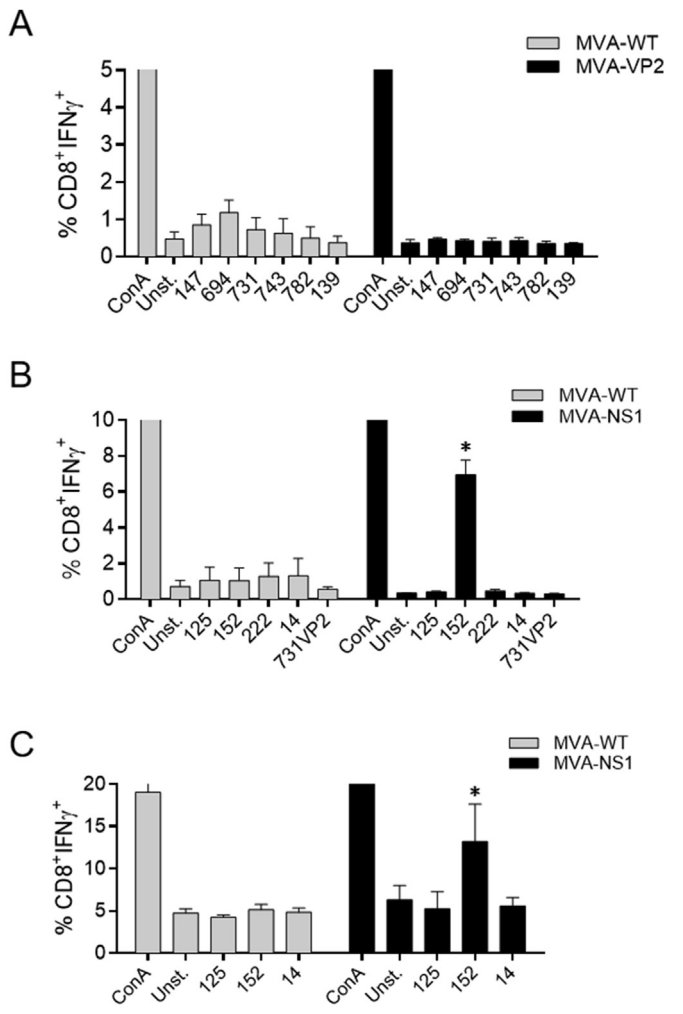

Fig. 4. Membrane labelling of CD107a and intracellular staining of IFN-γ in T CD8$^+$ cells of MVA-VP2 or MVA-NS1 immunized IFNAR(−/−) mice. Two weeks after the second immunization, spleens were harvested and the splenocytes were stimulated with 10 μg/ml of each peptide. Membrane CD107a and intracellular IFN-γ production was analyzed in CD8-positive cells by flow cytometry. Black bars: mice immunized with MVA-VP2 or MVA-NS1; grey bars: mice immunized with MVA-wt. The results represent the average of 4 mice ± SEM. Asterisks represent significant difference between samples, calculated by Man-Whitney non parametric test ($p \leq 0.05$).

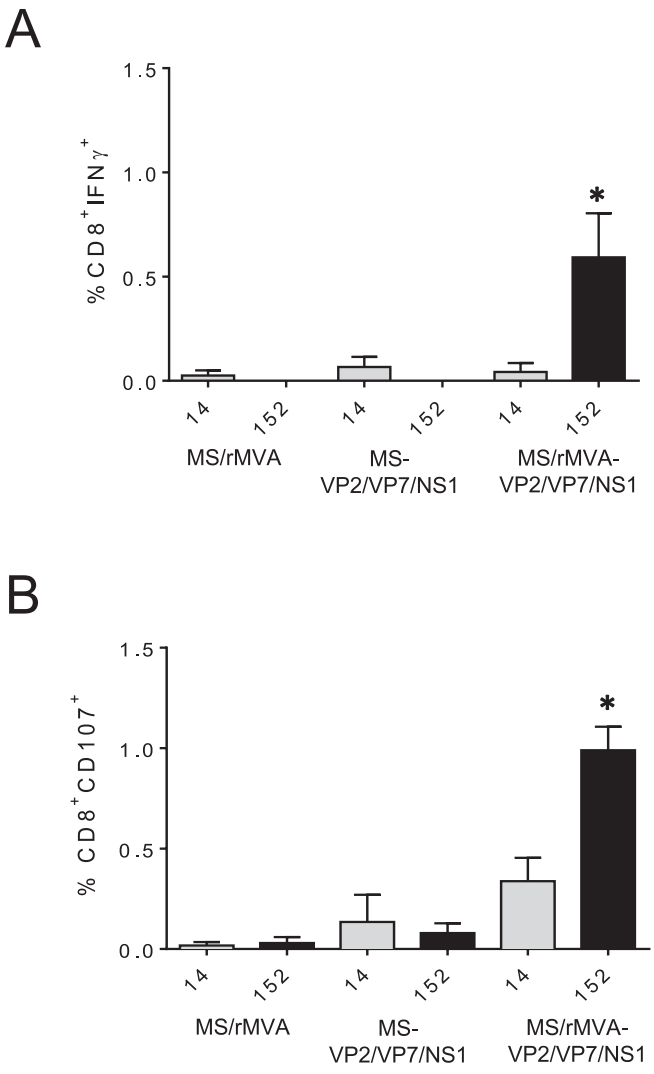

Fig. 5. Membrane labelling of CD107a and intracellular staining of IFN-γ in T CD8$^+$ cells of MS-VP2/VP7/NS1 or MS-rMVA-VP2/VP7/NS1 immunized IFNAR(−/−) mice. Two weeks after second immunization, spleens were harvested and the splenocytes were stimulated with 10 μg/ml of peptides 152 and 14 from NS1. At 5 h post-stimulation, intracellular IFN-γ production and labeling of CD107a were analyzed in CD8-positive cells by flow cytometry. Grey bars: peptide NS1-14; black bars: peptide NS-152. The results represent the average of 4 mice ± SD. Asterisks represent significant difference between immunized and non-immunized mice, calculated by Man-Whitney non parametric test ($p \leq 0.01$).

suggesting that the rMVA-VP2/VP7/NS1 booster stimulate a Th1-type immune response.

Antibody isotypes play different roles in antiviral immunity. Influenza vaccines that stimulate IgG1 and IgG2a antibody subclasses induce better protection that those that induce only IgG1 with neutralizing activity (Huber et al., 2006). IgG2A antibodies are better suited to induce antibody-mediated cell cytotoxic responses and complement mediated responses (Liu et al., 2016; Tjiam et al., 2015; Yendo et al., 2016) which have been shown to contribute to the clearance of viruses from infected hosts (Huber et al., 2001). Although there are not studies about the role of the different isotypes in the BTV protection, these should be addressed in order to rationally improve BTV vaccine strategies.

Cellular immune responses, specially CTL have been demonstrated to be important in the clearance of homologous and heterologous serotypes of BTV (Jeggo et al., 1985) and MVA has been described as a potent inductor of CD8 T cell responses when used as heterologous boost vaccination following a strong priming agent expressing the same antigen (Cottingham and Carroll, 2013; Whelan et al., 2009). Previous work in our laboratory using rMVA as a boost in the vaccination strategy showed that the heterologous DNA/rMVA prime-boost immunization expressing VP2, VP7, and NS1 BTV-4 proteins protected IFNAR(−/−) mice against heterologous challenges with BTV-1 and BTV-8, and reduced viraemia significantly in sheep infected with the heterologous BTV-8 (Calvo-Pinilla et al., 2012, 2014). In this work, ELISPOT assay showed that splenocytes of mice boosted with rMVA-VP2/VP7/NS1 elicited stronger cellular immune response than mice immunized twice with MS-VP2/VP7/NS1. To better analyze the cellular response phenotype induced by the BTV antigens included in the experimental vaccine and find immunodominant epitope(s) to CD8$^+$ T cell responses we studied *in silico* and *in vivo* the presence of CD8 epitopes in proteins VP2 and NS1 of BTV-4. Although CD4 and CD8 epitopes have been described for the VP7 protein of BTV-8 in mice and sheep (Rojas et al., 2011), we focused the study in proteins VP2 and NS1 where CTL epitopes have been described in BTV immunized sheep (Andrew et al., 1995; Janardhana et al., 1999). None of

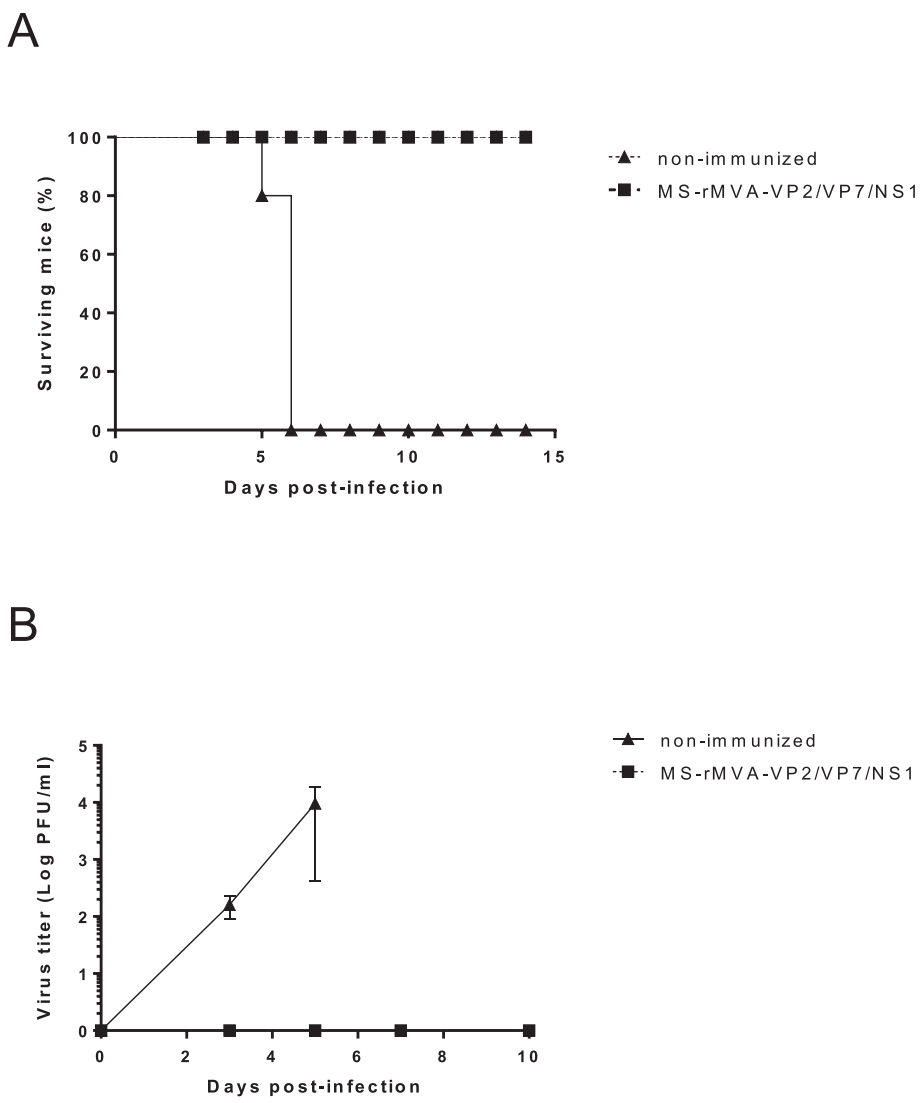

Fig. 6. Protection of MS-rMVA-VP2/VP7/NS1 vaccinated IFNAR(−/−) mice against a BTV-1 challenge. Mice (8 weeks old, 5 per group) were immunized twice by homologous prime-boost vaccination with MS-rMVA-VP2/VP7/NS1 administered 3 weeks apart. Non-immunized group was immunized with MS-rMVA and used as a control. Two weeks after the second immunization all mice were subcutaneously inoculated with 10^2 PFUs of BTV-1. (A) Survival rates of immunized and non-immunized IFNAR(−/−) mice after inoculation with BTV-1. The mice were observed every 24 h for 15 days. (B) Titers of BTV-1 recovered in blood of immunized and non-immunized IFNAR(−/−) mice after challenge. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars.

the six predicted VP2 peptides were capable of eliciting IFN-γ production in splenocytes from rMVA-VP2 immunized mice. In contrast, one peptide NS1-152 of NS1 protein was identified as a $CD8^+$ T cell epitope that induced the expression of IFNγ and CD107a in $CD8^+$ T cells, a marker of cytotoxic activity. Importantly, NS1-152 epitope is conserved among all BTV serotypes and it has been also previously characterized as a T cell epitope in sheep and C57BL/6 mice (Rojas et al., 2014).

Intracellular cytokine staining studies showed that peptide NS1-152 induced the activation of $CD8^+$ T cells and CTLs *in vivo* in animals boosted with rMVA-VP2/VP7/NS1, in contrast with previous observations where the immunization with MS-VP2/VP7/NS1 predominantly induces a $CD4^+$ T cell response (Marin-Lopez et al., 2014). In addition, 100% of the MS- rMVA-VP2/VP7/NS1 vaccinated IFNAR(−/−) mice survived to the challenge with the heterologous virus BTV-1 and viraemia was not observed in immunized animals after BTV-1 challenge.

In summary, the combination of microspheres and rMVA in a vaccination strategy improves the cellular immune response and cross-protection capacity of the experimental BTV subunit vaccine based on microspheres. We show that vaccination of IFNAR(−/−) mice with MS/rMVA expressing VP2, VP7, and NS1 proteins of BTV-4 achieves protective homotypic and heterotypic immunity and protection against homologous and heterologous infection with BTV-4 and BTV-1. This new experimental strategy is an attractive approach to generate new effective, safe and cross-protective marker vaccines again the multiple BTV serotypes.

Acknowledgments

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Estudio de la capacidad de la proteína no estructural NS1 de BTV, conservada entre los 27 serotipos descritos, como antígeno inductor de respuesta celular y protección multiserotipo frente al virus.

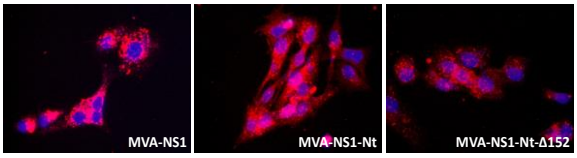
El desarrollo de vacunas frente a la lengua azul, enfermedad reemergente y prevalente de rumiantes, tradicionalmente se ha centrado en los antígenos de superficie del virus que lo provoca y que son los responsables de la inducción de anticuerpos neutralizantes. Debido a la variabilidad antigénica del virus, con 27 serotipos distintos descritos, estas vacunas son específicas de serotipo. En este trabajo mostramos cómo con una sola proteína del virus, la proteína no estructural NS1, expresada a través del vector viral recombinante MVA se consigue protección multiserotipo frente a 4 serotipos distintos del virus de la lengua azul (BTV-1, BTV-4, BTV-8 y BTV-16), tanto del topotipo *Western* como del *Eastern* y a un *reassortant* natural de BTV-1 y BTV-4 como es el virus BTV-4M. Esta protección es fuertemente dependiente de la respuesta celular T CD8 citotóxica y se produce en ausencia de anticuerpos neutralizantes. Hemos podido comprobar que su capacidad antigénica protectora reside en su región amino-terminal (aminoácidos 1-270), generando el vector rMVA-NS1-Nt, el cual alcanza los mismos niveles de protección que el vector rMVA-NS1, a través de la activación de una respuesta celular T CD8 citotóxica. Para conseguir la activación de esta respuesta T CD8 protectora es necesario la presencia de un epítopo T CD8 inmunodominante localizado en dicha región (GQIVNPTFI), ya que su deleción en la secuencia de aminoácidos conlleva la pérdida de su capacidad protectora como vacuna. Estos resultados revelan la importancia de la proteína NS1 en cuanto a protección mediada por células T CD8+ frente a múltiples serotipos de BTV cuando es vehiculizada por medio de un vector viral vacunal como el MVA recombinante.

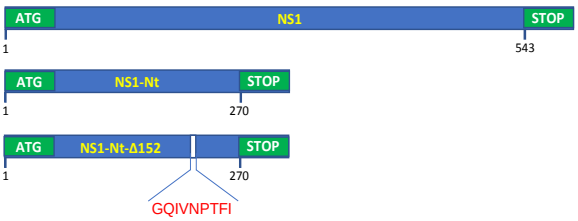
rMVA-NS1

rMVA-NS1-Nt

BTV-1,BTV-4, BTV-4M, BTV-8 Y BTV-16 → Protección frente a todos los serotipos estudiados de BTV
Ausencia Abs neutralizantes
Activación respuesta celular T CD8 citotóxica

MVA NS1-NtΔ152 **BTV-4** → Pérdida de la capacidad protectora
Ausencia Abs neutralizantes
Pérdida de la activación respuesta celular T CD8 citotóxica → Péptido 152 inmunodominante





IMMUNOPROTECTION AGAINST MULTIPLE SEROTYPES OF BLUETONGUE VIRUS INFECTION IS DEPENDENT ON CD8 T CELL RESPONSES TO AN IMMUNODOMINANT EPITOPE WITHIN THE NON-STRUCTURAL PROTEIN NS1

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SUMMARY

The development of vaccines against Bluetongue, a prevalent livestock disease, has been focused on surface antigens that induce strong neutralizing antibody responses. Because their antigenic variability, these vaccines are usually serotype restricted. We now show that a single highly conserved non-structural protein, NS1, expressed in a modified vaccinia Ankara virus (MVA) vector can provide multiserotype protection against Bluetongue virus that is largely dependent on CD8 T cell responses. We found that the protective antigenic capacity of NS1 resides within the N-terminus of the protein and is provided in the absence of neutralizing antibodies. The protective CD8 T cell response requires the presence of a specific peptide within the N-terminus of NS1, since its deletion ablates the efficacy of the vaccine formulation. These data reveal the importance of the non-structural protein NS1 in CD8 T cell-mediated protection against multiple BTV serotypes when vectorized as a recombinant MVA vaccine.

INTRODUCTION

Bluetongue virus (BTV) causes a hemorrhagic disease of ruminants that is transmitted by *Culicoides* species of biting midges. To date, 27 serotypes of BTV have been identified (Zientara, Sailleau et al. 2014) with two more putative serotypes and several other variants being further described (Wright 2014; Jenckel, Breard et al. 2015; Maan, Maan et al. 2015; Schulz, Breard et al. 2016; Mayo, Lee et al. 2017). Because of supportive environmental conditions, BTV has been historically prevalent in tropical and subtropical regions located between 35 ºS and 45 ºN, coinciding with the presence of competent *Culicoides* vectors (Maclachlan, Mayo et al. 2015). However, outbreaks have been reported further North, including in several countries in Europe, Asia, Oceania and the Americas. Since 1998, BTV serotypes 1, 2, 4, 6, 8, 9, 11 and 16 have been introduced in Europe while additional novel serotypes have recently invaded historically-endemic countries such as Israel, Australia and the USA. Five BTV serotypes have long been recognized as enzootic in North America. Since 1998 ten additional previously exotic serotypes have been isolated in south-eastern USA; and most recently, BTV infection of sheep was detected for the first time in Ontario, Canada in 2015, which represents the furthest North expansion of BTV in North America (Mayo, Lee et al. 2017). Worldwide, BTV has been estimated to cause direct (disease) and indirect (trade, vaccines, etc.) losses of over $3 billion per year (Tabachnick, Robertson et al. 1996; Mellor, Carpenter et al. 2008).

The development of an effective vaccine remains an important goal for the safe and cost-effective control of this disease. Bluetongue vaccine development has classically focused on inactivated and attenuated virus. However, live attenuated viral vaccines are associated with clinical signs, viremia compatible with transmission and risk of gene segment reassortment (Batten, Maan et al. 2008; Marín-López, Barriales et al. 2016). Moreover, these vaccines are serotype-specific, inducing neutralizing antibodies against the outer capsid protein VP2. Although conventional vaccines have controlled or limited BTV expansion in the past, they cannot address the need for cross-protection among serotypes and do not allow to distinguish between infected and vaccinated animals (DIVA strategy). Therefore, the generation of universal vaccines that induce effective protection against multiple virus serotypes is an increasingly pressing goal, especially since more than one BTV serotype circulates in all regions of the world where BTV is stably endemic.

Vaccines against BTV have commonly been aimed at the induction of broadly neutralizing antibody and T-cell responses, since both arms of the adaptive immune response have a role in protection against BTV (Jeggo, Wardley et al. 1984) (Stott, Osburn et al. 1979; Roy, Bishop et al. 1994). The non-structural (NS) proteins, NS1, NS2, NS3/3A, NS4 and NS5, play a number of important

roles in virulence, viral replication, maturation, and export, suggesting that NS proteins are candidate targets for antiviral therapies (Owens, Limn et al. 2004; Ratinier, Caporale et al. 2011; Matsuo and Roy 2013; Stewart, Hardy et al. 2015). NS1 is the most highly synthesized viral protein in BTV-infected cells and is highly conserved among different serotypes (Urakawa and Roy 1988; Van Dijk and Huismans 1988; Owens, Limn et al. 2004; Schwartz-Cornil, Mertens et al. 2008). This protein contains epitopes associated with both T-cell and humoral responses, and antibody responses against NS1 protein may be important contributors to immune protection (Owens, Limn et al. 2004; Anderson, Breard et al. 2014; Rojas, Pena et al. 2014).

The use of viral vaccine vectors, such as Modified Vaccinia Ankara virus (MVA), deployed in heterologous prime-boost regimes, have been routinely developed to induce strong T cell responses targeting intracellular pathogens (Drexler, Staib et al. 2004). In fact, the heterologous prime-boost immunization using either DNA-MVA or muNSMi-microspheres-MVA expressing the structural proteins VP2 and VP7 confer total protection in the IFNAR(-/-) mice model against heterologous challenges when NS1 is included in the vaccine composition (Calvo-Pinilla, Navasa et al. 2012; Marin-Lopez, Calvo-Pinilla et al. 2017). We have developed a safe, DIVA and universal experimental vaccine against BTV based on the recombinant viral vector MVA expressing NS1 or a truncated version of the protein (MVA-NS1-Nt). We show that cytotoxic CD8 T cell responses against NS1 provide essential help to provide protection against lethal challenge with several BTV serotypes. Furthermore, we demonstrate that the specific T CD8 epitope, NS1-152, is essential in order to elicit protection, since its deletion abolishes the protective capacity of this vaccine.

RESULTS

An MVA-NS1 recombinant vaccine protects against challenge with multiple BTV serotypes.

Adult IFNAR(-/-) mice were immunized with MVA-NS1 by intraperitoneal injection in a prime-boost regimen at three-week intervals. Two weeks after the second immunization, the mice were challenged subcutaneously with lethal doses of several BTV serotypes, including BTV-4M, BTV-1, BTV-8, BTV-4 or BTV-16. Survival, viremia, clinical signs and hematological parameters were then analyzed. After five days, all non-immunized animals had succumbed to the infection, independently of serotype, except for one mouse that survived BTV-16 infection. In contrast, all immunized mice survived infection, except one mouse challenged with BTV-4M that died at 5 d.p.i. (Figure 1A).

We determined viremia after immunization and challenge by virus isolation on cell culture from whole blood for BTV-1, BTV-4 and BTV-8 and by RT-qPCR for BTV-4M and BTV-16, due to the inability to these serotypes to form lysis plaques. Viremia was detected at 3 d.p.i. and the presence of virus in blood increased thereafter until sacrifice in non-immunized and infected animals, with titers up to $9.3x10^3$, 33 and 5.10^3 PFU/ml by plaque assay for BTV-1, BTV-4 and BTV-8 respectively (Figure 1B). In contrast, no immunized mice showed viremia by plaque assay after challenge. We also analyzed by RT-qPCR the presence of BTV genomes in the blood of immunized and non-immunized IFNAR(-/-) mice challenged with BTV-4M and BTV-16. BTV-4M genomes were readily detected in non-immunized mice at day three of infection (C*t* mean: 32.7) and increased (C*t* mean: 28.7) thereafter until sacrifice. In contrast, the RT-qPCR reaction yielded negative results for the majority of the immunized mice at all analyzed days post-challenge (*C*t≥38), except one immunized mouse that died (*Ct* mean: 28.4) and other that survived the challenge (*Ct*: 34.89) (Figure 1C left). For BTV-16 infection, we detected BTV-16 genomes at 4 d.p.i. (*Ct* mean: 30.8), with the highest level of BTV-16 genomes at day 6 post infection (*Ct* mean: 25.08) decreasing at 10 d.p.i. (*Ct* mean: 29.5) before the death of the animals in the non-immunized group. However, immunized animals had negative or low viremia, reverting to negativity during the course of the infection (*Ct* means of 34.9, 36.7 and 36.8 for 4, 6 and 10 d.p.i. respectively). (Figure 1C right).

All non-immunized and infected mice presented clinical signs (Figure 1D). They were similar in all challenges with the different BTV serotypes, presenting rough hair coat, lethargia, eye swelling and hypothermia, as opposed to the immunized mice where clinical signs were not detected. In the case of non-immunized and BTV-16 challenged animals, some animals developed hind leg paralysis at day 10 post infection.

We then determined hematological changes in mice after BTV infection. We evaluated blood levels of neutrophils, lymphocytes, monocytes and platelets in infected mice at 3 and 5 d.p.i. for BTV-

1, BTV-4, BTV-4M and 4, 6, 8 and 12 d.p.i. for BTV-16 (measures of 12 d.p.i. in the case of non-immunized and infected animals corresponded to the unique survivor of this group, represented as R in the S3 graph). BTV-4 infection resulted in a 10-fold drop in the absolute lymphocyte count, accompanied by a significant decrease in the levels of monocytes as well as a 5-fold decrease in the number of platelets (Figure 1E). These results were similar for all the BTV serotypes used in the challenges, with exceptions, as the increase in the platelet level in non-immunized and BTV-16 infected animals at 10 d.p.i., possibly due to coagulation disorders (Figure S3). Regarding the quantity of neutrophils, no significant differences were observed in neutrophil counts in BTV-4-infected mice, possibly due to the faster pathology of this serotype (Figure 1E). In contrast, a significant increase was observed in BTV-1, BTV-8 and BTV-16-infected non-immunized animals (Figure S3). MVA-NS1 immunized animals did not show variation on neutrophil levels after challenge, except those infected with BTV-4M, where an increase in the level of neutrophils was observed at 3 d.p.i., although this value turned to normal at 5 d.p.i. (Figure S3).

All these data indicate that the immunization of mice with MVA-NS1 confers protection against multiple BTV serotypes and reduces or abrogates viremia and clinical signs, while maintaining normal blood parameters.

MVA-NS1 immunization generates strong CD8 T cell and non-neutralizing antibody responses.

In order to analyze the specific immune responses elicited in mice immunized with MVA-NS1 we developed ELISPOT, intracellular cytokine staining assay (ICS), virus neutralization tests (VNT) and ELISA assays. Re-stimulation of splenocytes with recombinant NS1 protein yielded detectable specific IFNγ-producing cells (mean: 100) in MVA-NS1 immunized mice but not in non-immunized animals (mean: 6.3) (Figure 3C). We have recently determined that peptide 152 (GQIVNPTFI) is an immunodominant CD8 T cell epitope from NS1. We therefore, re-stimulated splenocytes with the peptide p152 and a non-relevant peptide (p14, Control) and determined intracellular IFNγ production and the CD107 cytotoxic marker expression in CD8 T cells. We observed a significant induction of CD8+IFNγ+ and CD8+CD107+ cells (P: 0.057) upon re-stimulation of MVA-NS1 immunized mouse splenocytes with p152 (Figure 2C, D). In contrast, the re-stimulation of splenocytes from control MVA-wt-immunized mice showed negligible responses to the peptide (Figure 2C, D). These data confirm that immunization with MVA-NS1 elicits a cytotoxic T CD8 cellular response in mice, including to the immunodominant peptide p152.

We next performed an ELISA assay to detect NS1-specific antibodies in the sera of the immunized animals. Significant titers of NS1-specific antibodies were detected in MVA-NS1-

immunized mice (O.D. mean: 1.26) (Figure 2A). Due to the commercially available diagnostics test for BTV are based on the VP7 protein, these results suggest that an ELISA diagnosis system based on recombinant NS1 recognition could be a good tool to discern between infected and MVA-NS1 vaccinated animals. In order to determine whether immunization with MVA-NS1 induced the production of neutralizing antibodies, we performed a VNT assay. Although NS1 induced high levels of antibodies, only negligible levels of neutralizing antibodies were detected in the sera of both immunized and non-immunized animals (Figure 2B).

Overall, our results show that a recombinant MVA-NS1 vaccine induces a potent and protective CD8 T cell immune response in the absence of neutralizing antibodies that are nevertheless amenable to be used as a tool to distinguish vaccinated and infected animals.

NS1-specific CD8 T cells confer partial protection against BTV challenge.

Our results showed that protection elicited by NS1 relies on the immunodominant peptide p152. In order to assess whether a p152-specific CD8 T cell response is sufficient to confer protection against challenge, we adoptively transferred purified CD8 T cells from IFNAR(-/-) mice immunized with MVA-NS1, using MVA-wt as a negative control. MVA-NS1 or MVA-wt immune CD8 T cells were transferred to naive recipient IFNAR(-/-) mice and challenged simultaneously with a lethal dose of BTV-4. Two different doses of transferred CD8 T cells were assayed. Transfer of 10^6 CD8 T cells from MVA-NS1 immunized animals to naïve and infected animals did not show significant differences in survival, clinical signs or viremia, and all animals died (Figure 6A left, B left, C left). In contrast, when the dose administered was increased to $6x10^6$ CD8 T cells from MVA-NS1 immunized animals, a statistically close to significant difference was observed, with a delay in mortality and in the appearance of clinical signs (Figure 6A right, B right). Control mice transferred with CD8+ cells from MVA-wt immunized animals started to die at 4 d.p.i., while mice transferred with MVA-NS1 immune CD8 T cells succumbed 24 hours later. No viremia was detected in mice transferred with MVA-NS1 immune CD8 T cells at the days analyzed (Figure 6C). These results demonstrate an important role for NS1-specific CD8 T cells in controlling BTV infection in mice.

To check whether anti-NS1 serum is involved in protection, heat-inactivated sera of non-immunized and immunized animals were also transferred intraperitoneally into naïve mice (200µl/animal), and challenged after sera transfer. All animals died (between 4 and 5 d.p.i.) and non-significant differences were observed between these groups (Figure 6 D).

The N and C terminal regions of NS1 elicit distinct immune responses.

According to the Immuno Epitope Data Base (IEDB) (Kolaskar & Tongaonkar Antigenicity Results), the most antigenic residues of NS1 are located in the C proximal region (NS1-Ct), while the amino proximal region (NS1-Nt) contains the most hydrophobic residues. We have reported that the most probable theoretical NS1 CD8 T epitopes are found in the NS1-Nt region (Marin-Lopez, Calvo-Pinilla et al. 2017) . We generated recombinant MVAs expressing NS1-Nt (MVA-NS1-Nt) and NS1-Ct (MVA-NS1-Ct). Mice were immunized with MVA-NS1-Nt, MVA-NS1-Ct or inoculated with MVA-wt as described above and antigen specific immune responses were assayed by ELISA, VNT and ELISPOT. Sera from immunized mice were analyzed for the presence of specific IgG antibodies against NS1. High titers of antibodies were observed in sera of MVA-NS1-Ct-immunized mice (O.D. mean: 0.89) (Figure 3A). In contrast, sera from MVA-NS1-Nt immunized mice and IFNAR(-/-) mice inoculated with MVA-wt showed low antigen-specific antibody titers (O.D. mean: 0.22 and 0.36 respectively). These result support the *in silico* analysis*,* where NS1-Ct encompasses the more antigenic region of NS1. A VNT test was also performed to ensure the absence of neutralizing antibodies (Figure 3B). To further analyze the cellular immune response that these fragments confer, we determined the amount of IFNγ produced by the cells after being stimulated with recombinant NS1 protein in an ELISPOT test. After stimulation, MVA-NS1-Nt immunized mice developed detectable specific IFNγ-producing cells (mean: 65.3), when compared to the MVA-NS1-Ct immunized and non-immunized splenocytes (mean: 23.3 and 6.3 respectively) (Figure 3C). The increase in the level of IFNγ producing cells after immunization with MVA-NS1-Nt was significant when was analyzed by Student's t-test compared with the non-immunized mice but not when using MVA-NS1-Ct as inmunogen. Furthermore, the peptide p152 induced a significant response in CD8 T cells by intracellular determination of IFNγ production, The presence of the cytotoxic marker, CD107 was close to significant (P:0.057) (Figure 3D, E) in splenic cells from MVA-NS1-Nt immunized mice, while no response was detected in splenocytes from animals inoculated with MVA-wt. These data suggest that the strategy of immunization based on MVA-NS1-Nt achieves similar response levels that those by MVA-NS1 and elicits an immune T CD8 cellular response in the animal model.

These results show that MVA-NS1-Ct elicits a potent, albeit non-neutralizing humoral immune response, whereas MVA-NS1-Nt promotes the activation of cytotoxic CD8+ cells, in part through the peptide p152.

NS1-Nt immunization mimics the multiserotype protective effect of NS1.

We then determined the immunoprotective capacity of immunization regimes with both MVA-NS1-Nt and MVA-NS1-Ct. Mice were immunized as before with MVA-NS1-Nt and MVA-NS1-Ct

and challenged with a lethal dose of BTV-4. A delay, albeit not significant (p = 0.11), in the mortality of animals immunized with MVA-NS1-Ct was observed when compared with the non-immunized group. Some animals developed viremia and presented clinical signs (Figure 4A left, B). In contrast, all mice immunized with MVA-NS1-Nt were protected during the infection, in absence of viremia and clinical signs (Figure 4A right, D, F). When we evaluated the potential use of MVA-NS1-Nt as a multiserotype vaccine, we observed that all MVA-NS1-Nt-immunized mice survived BTV infections regardless of their serotype, except for one mouse challenged with BTV-1 that died at 4 d.p.i. (Figure 4C). No viremia was detected in the blood of the immunized animals in the case of BTV-1 and BTV-8 by plaque assay (Figure 4D), and negative or reduced levels in the case of BTV-4M and BTV-16 respectively, by RT-qPCR (Figure 4E left and right respectively). The evaluation of blood levels of neutrophils, lymphocytes, monocytes and platelets at 3 and 5 d.p.i, showed that all these parameters remained within standard levels in the immunized animals for all the infections (Figure 4G and S3).

These data indicate that immunization with MVA-NS1-Nt confers protective immunity against multiple BTV serotypes, almost completely abrogating viremia, and while maintaining normal blood parameters, mimicking immunization with the full protein.

Peptide 152 confers protective capacity to NS1-Nt.

In order to address the role of peptide p152 on the immunoprotection elicited by NS1-Nt, we generated a recombinant MVA that expressed NS1-Nt with a deletion on this peptide (MVA-NS1-NtΔ152). We then evaluated survival, viremia and clinical signs in animals immunized with this viral vector and subsequently challenged with BTV-4. A small, but not significant delay in the mortality of mice immunized with MVA-NS1-NtΔ152 was observed compared to the non-immunized group (P: 1.00) (Figure 5A). Viremia was detectable at day 3 and 5 and a delay in the appearance of clinical signs was also observed (Figure 5B, C). Concerning the blood parameters, we observed a decrease of lymphocytes, monocytes and platelets and an increase of neutrophils in animals immunized with MVA-NS1-NtΔ152 and infected with BTV-4, typical of non-immunized and infected individuals (Figure 5D). We also checked the absence of neutralizing antibodies in the sera of immunized animals (Figure 5E) and analyzed the phenotype of the BTV-specific IFNγ producing cells after immunization. No induction in the percentage of CD8+IFNγ+ and CD8+CD107+ cells was observed in splenic cells from MVA-NS1-NtΔ152 immunized mice when they were re-stimulated with p152 as we expected, due to the deletion of p152 in the sequence of NS1-Nt (Figure 5F, G).

These data demonstrate that within the N terminus of NS1, the immunodominant peptide p152 is required for full protection against BTV challenge.

DISCUSSION

Protection against viral infection depends on the action of several immune effector mechanisms. For example, the presence of a strong type I interferon response is essential to blunt the initiation of infection, as demonstrated in strains of mice that are able to signal through IFNAR (Calvo-Pinilla, Rodriguez-Calvo et al. 2009). For vaccine development, targeting the appropriate acquired immune response is critical for a successful protective effect. In the case of Bluetongue, the generation of strong neutralizing antibodies against capsule antigens, such as VP2, provides full protection (Parker, Herniman et al. 1975; Huismans, van der Walt et al. 1987; Calvo-Pinilla, Rodriguez-Calvo et al. 2009; Stewart, Dubois et al. 2013; Kochinger, Renevey et al. 2014; Marin-Lopez, Otero-Romero et al. 2014; Nunes, Hamers et al. 2014; Legisa, Perez Aguirreburualde et al. 2015). Protection mediated by neutralizing antibodies has also been demonstrated for other orbivirus, such as African Horse Sickness virus (Calvo-Pinilla, de la Poza et al. 2015) and other emerging viral infections, including Zika, Ebola, Rift Valley Fever or Yellow Fever viruses (Stokes A. 1997; Sapparapu, Fernandez et al. 2016; Warimwe, Gesharisha et al. 2016; Chen, Li et al. 2017; Stokes 1997). However, because surface antigens are highly variable among serotypes, vaccines based on these proteins are specific to the serotype they target and provide a poor cross-protection capacity. In order to confer multiserotype protection against BTV, CD8 T cell responses are required, probably because the antigens at which they are directed are highly conserved among serotypes (Jones, Chuma et al. 1996; Anderson, Breard et al. 2014; Anderson, Hagglund et al. 2014). Herein, we demonstrate the multiserotype protective capacity of MVA-based vaccines expressing the non-structural protein NS1. Furthermore, the level of protection and induction of cytotoxic CD8 T cell activity is mimicked by the N terminal domain of NS1 (NS1-Nt) in the absence of neutralizing antibodies. Remarkably, we pinpoint the protective capacity of this antigen to the presence of a CD8 T cell specific epitope within NS1-Nt.

NS1 is one of the major immunogens for CD8 T cells (Anderson, Breard et al. 2014; Anderson, Hagglund et al. 2014). The primary antigenic site of NS1 has been localized within the carboxyl terminus, NS1-Ct (Monastyrskaya, Gould et al. 1995); however, we identified the majority of theoretical T CD8 cell epitopes within the amino terminus of the protein (NS1-Nt). Indeed, our results show that while a recombinant MVA containing NS1-Ct (MVA-NS1-Ct) is able to induce high antibodies titers, it does not induce significant levels of IFNγ-secreting CD8 T cells. Importantly, the immunization with MVA-NS1-Ct does not confer protection against a lethal challenge, although it induces a delay in the mortality rate of the mice.

In contrast to NS1-Ct, immunization with MVA-NS1-Nt leads to survival upon infection against multiple serotypes of BTV. Immunization with MVA-NS1-Nt leads to low antibody titers with no neutralizing activity that are not able to induce protection when passively transferred to naïve mice before challenge with BTV. However, immunized mice develop strong cytotoxic CD8 T cell responses against this antigen. Importantly, the transfer of CD8 T cells from MVA-NS1-Nt-immunized mice to naïve animals induces protection against challenge with the virus. Our data demonstrate that the protective capacity of the vaccine based on NS1-Nt is due to the presence of the epitope, p152. Indeed, the deletion of this CD8 T cell epitope almost completely abrogates the protective effect elicited by MVA-NS1-Nt, albeit a small delay in the infection also occurs in its absence. These results demonstrate that the amino terminal region of NS1 (NS1-Nt) is sufficient to induce protection against multiserotype BTV challenge through the induction of cytotoxic CD8 T cells that is largely dependent on the presence of the epitope p152.

Bluetongue is an important livestock disease worldwide. In order to control BTV expansion, the development of an efficient multiserotype vaccine that allows the differentiation between infected and vaccinated animals while protecting against all serotypes is needed. Our results show that a single highly conserved antigen expressed in a MVA vector can provide significant multiserotype protection against BTV that is largely dependent of a single CD8 T cell epitope. These data reveal the importance of the non-structural protein NS1 in CD8+ T cell-mediated protection against multiple BTV serotypes, when vectorized as a recombinant MVA vaccine. An ELISA diagnosis system based on recombinant NS1 recognition, in combination with the commercial ELISA BTV diagnostic test based on the protein VP7 could be a good tool to discern between naturally infected and MVA-NS1 vaccinated animals. Overall, these data demonstrate that the development of vaccines that can induce strong CD8 T cell responses against BTV based on the 152 peptide could accomplish maximal protective efficacy.

STAR★ METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, figures and tables.

AUTHOR CONTRIBUTIONS

A.M.L. designed and performed the experiments, analyzed the data, and wrote the manuscript;

E.C.P., D.B. and G.L Designed and performed the experiments;

A.B. conceived the project, and conceptual advice;

J.O. conceived the project, designed the experiments, and wrote the manuscript.

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SUPPLEMENTAL DATA

IMMUNOPROTECTION AGAINST MULTIPLE SEROTYPES OF BLUETONGUE VIRUS INFECTION IS DEPENDENT ON CD8 T CELL RESPONSES TO AN IMMUNODOMINANT EPITOPE WITHIN THE NON-STRUCTURAL PROTEIN NS1

Alejandro Marin-Lopez, Eva Calvo-Pinilla, Diego Barriales, Gema Lorenzo, Alejandro Brun, and Javier Ortego.

STAR★METHODS.

KEY RESOURCES TABLE.

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-Mouse CD8a PerCP-cyanine 5.5	eBioscience	REF 45-0081-82 LOT E08300-1634
Anti-Mouse IFN gamma PE	eBioscience	REF 12-7311-82 LOT E02135-1633
Anti-Mouse CD107a FITC	Miltenyi Biotec	REF 130-102-191 LOT 5151117252
Goat anti-Mouse PO	Sigma-Aldrich	REF A4416
Alexa Fluor 594 goat anti-Mouse IgG	Life Technologies	A11005
Bacterial and Virus Strains		
BTV-1	The Pirbright Institute	ALG2006/01

BTV-4	The Pirbright Institute	SPA2004/02
BTV-4M	The Pirbright Institute	SPA2009/09
BTV-8	The Pirbright Institute	BEL2006/01
BTV-16	The Pirbright Institute	RSArrrr/16
MVA-wt	Dr Francisco Rodriguez, Spanish National Center for Biotechnology (CNB-CSIC)	
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
Recombinant NS1	Our laboratory	
NS1-152	Proteogenix	LOT P140404-XL394499
NS1-14	Proteogenix	LOT P140404-XL 394501
Concanavalin A	Sigma-Aldrich	REF C2272
Brefeldrin A	Sigma-Aldrich	REF B6542-5MG LOT 091M4000V
Critical Commercial Assays		
CD8a mouse+ T Cell Isolation Kit Miltenyi Biotec	Miltenyi Biotec	Order no. 130-104-075
Deposited Data		

Experimental Models: Cell Lines		
DF-1 cells	ATCC	Cat. No. CRL-12203
Vero cells	ATCC	Cat. No. CCL-81
Experimental Models: Organisms/Strains		
A129 (IFNa/b R -/-); (129 SvEv, inbred).	B&K Universal Ltd UK	Science 264:1918-1921
Oligonucleotides		
NS1 SmaI Fw 1 CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC	Sigma-Aldrich	N/A
NS1 SmaI Rs 786 CGCCCGGGCTAACCTGTCGGAACCTTTCCAAAAAG	Sigma-Aldrich	N/A
NS1 SmaI Fw 811 CGGAATTCATGGAATTTCCATTACATCAGATGATGC	Sigma-Aldrich	N/A
NS1 SmaI Rs 1628 (1659) CGCCCGGGCTAATACTCCATCCACATCTG	Sigma-Aldrich	N/A
NS1 Δ152 Fw AATCAAAGAATCAAGATATCGCCAAATTGCATACTATTTC	Sigma-Aldrich	N/A
NS1 Δ152 Rs GATATCTTGATTCTTTGATTGGCACATAGATGTAAGGCAT	Sigma-Aldrich	N/A
NS1 152 Fw CCCGGG ATG CCT TAT ATC TAT GTA CCA GTC	Sigma-Aldrich	N/A
NS1 152 Rs GCCCCGGGCTAGTGGTGGTGGTGGTGGCGATATCTTG ATATAAATGTTGG	Sigma-Aldrich	N/A
Recombinant DNA		

pSC-11	Dr Francisco Rodriguez, Spanish National Center for Biotechnology (CNB-CSIC)	Mol. Cell. Biol. 5:3403-3409 (1985)
pSC-11-NS1-Nt	This paper	N/A
pSC-11-NS1-Ct	This paper	N/A
pSC-11-NS1-NtΔ152	This paper	N/A
pSC-11-152	This paper	N/A
Software and Algorithms		
GraphPad Prism	GraphPad	https://www.graphpad.com/ scientific-software/prism/
FlowJo	FlowJo®	https://www.flowjo.com/solutions/flowjo
Lasergene-MegAlign	DNASTAR	https://www.dnastar.com/t-megalign.aspx
Other		

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statement

Animal experimental protocols were approved by the Ethical Committee of the Center for Animal Health Research (CISA-INIA) (Permit number: PROEX 037/15) in strict accordance with Spanish National Royal Decree (RD1201/2005) and international EU guidelines 2010/63/UE about protection of animals used for experimentation and other scientific purposes and Spanish Animal Welfare Act 32/2007. All work with infected animals was performed in a BSL3 laboratory of the Center for Animal Health Research (CISA-INIA).

Cells and viruses.

Chicken embryo fibroblasts (DF-1) (ATCC, Cat. No. CRL-12203) and Vero (ATCC, Cat. No. CCL-81) cells were grown in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 2mM glutamine, 10% heat-inactivated fetal bovine serum (FBS) and antibiotics. BTV serotype 1 (ALG2006/01) (BTV-1), BTV serotype 4 (SPA2004/02) (BTV-4), BTV serotype 4 *Morocco* strain (MOR2009/09) (BTV-4M), BTV serotype 8 (BEL/2006) (BTV-8) and BTV serotype 16 (RSArrrr/16) (BTV-16) were used in the experiments. BTV-1, BTV-4, BTV-8 and MVA virus stocks and titrations were performed as previously described (Calvo-Pinilla, Rodriguez-Calvo et al. 2009). BTV-4(M) and BTV-16 titrations were performed by RT-qPCR as previously described (Toussaint, Sailleau et al. 2007). *NS1 sequence alignment and the percentage of all serotypes of BTV used in this experiment are shown in S2.*

Animals and immunizations.

IFNα/ßR$^{o/o}$ IFNAR(-/-) 129/Sv mice and were purchased from B&K Universal Ltd UK. Eight-week old male mice were used throughout. Upon reception, the mice were held for 7 days for acclimatization under pathogen-free conditions in the biosafety level 3 (BSL3) animal facility at Center for Animal Health Research (INIA-CISA), Madrid. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the ethical review committee at the INIA-CISA and Comunidad de Madrid (Permit number: PROEX 037/15).
Groups of four/five IFNAR(-/-) mice were immunized by homologous prime boost vaccination with recombinant MVAs expressing NS1, NS1-Nt, NS1-Ct, NS1-NtΔ152 and NS1-152 peptide of BTV-4 or MVA wild type (wt) (non-immunized mice), administered 3 weeks apart. 10^7 PFUs of each rMVA construct were inoculated intraperitoneally.

METHOD DETAILS

Generation of recombinant MVAs.

The generation of MVA-NS1 have been previously described (Calvo-Pinilla, Navasa et al. 2012; Marin-Lopez and Ortego 2016). To generate MVA-NS1-Nt and MVA-NS1-Ct, a primer set targeting N-terminal region of NS1 (1 to 270 aa) (NS1 SmaI Fw 1, NS1 SmaI Rs 786) and another primer set targeting C-terminal region of NS1 (271 to 543 aa) (NS1 SmaI Fw 811, NS1 SmaI Rs 1628) was used to construct the transfer vectors pSC11-NS1-Nt and pSC11-NS1-Ct from pSC11-NS1. 152-deletion mutant NS1-Nt MVA (MVA-NS1-NtΔ152) were designed. To generate the deletion, oligonucleotide primers NS1 SmaI Fw 1 and NS1 Δ152 Rs, deleting a nine aa mutation (GQIVNPTFI), were used to

generate a PCR product from nucleotides (nt) 1 to 456 of the NS1 gene. The primers NS1 Δ152 Fw, including the deletion and NS1 SmaI Rs 786 were used to generate a PCR product from nt 485 to 786 of the NS1 gene. Both overlapping PCR products were used as templates for PCR amplification using the primers NS1 SmaI Fw 1 and NS1 SmaI Rs 786.The amplified DNA was digested with SmaI and cloned into the SmaI-digested pSC11 to obtain the pSC11-NS1-NtΔ152. To generate MVA-152, a recombinant MVA that encodes the aminoacid sequence MPYIYVPVKEGQIVNPTFISRYRHHHHHH, a set of primers was used (NS1 SmaI 152 Fw and NS1 SmaI 152 Rs).This sequence corresponds to peptide 152, flanked by 10 aa upstream and ten aa downstream, including a His tag (peptide 152 aa sequence underlined). All the sequences of the primers used in are described in table A in S1. *See the MVA expression by indirect immunofluorescence in S1 (B).*

Humoral immune response assays.

The Virus Neutralization Test (VNT) was used to determine neutralizing antibody titers against BTV-4. For plaque reduction assays, 2-fold dilutions of sera were mixed with 100 PFU of BTV-4, incubated for 1 h at 37 ºC and then plated into monolayers of Vero cells. After 1 h, agar overlays were added and the plates were incubated for 5 days. The titer was determined as the highest dilution that reduced the number of plaques by 50%.

Serum was analyzed for antibodies by ELISA as previously described (Calvo-Pinilla, Rodriguez-Calvo et al. 2009). Recombinant NS1 protein were adsorbed to 96-well Nunc-Immuno Maxisorp plates at a concentration of 150 ng/µL in Carbonate/Bicarbonate buffer. Briefly, plates were washed with PBS containing 0.05% Tween 20 (PBS/T) and blocked with 5% skimmed milk powder in PBS/T. Sera were diluted to 1:50, added in duplicate wells. Bound antibodies were detected using alkaline phosphatase-conjugated rabbit anti-mouse total IgG (Biorad, USA). Plates were developed by adding 3, 3', 5, 5'-Tetramethylbenzidine (TMB) substrate. Optical density was read at 450 nm (OD).

Ex vivo IFNγ ELISPOT and Flow cytometric analysis.

Groups of IFNAR(-/-) mice (n=4) were immunized following a homologous prime-boost regimen with rMVA-NS1, rMVA-NS1-Nt, rMVA-NS1-Ct¸ rMVA-NS1-NtΔ152, rMVA-152 or MVA-wild type (non-immunized group) three weeks apart. All animals were sacrificed at 10 days post-booster and their spleens were harvested for analysis by ELISPOT and intra-cellular cytokine staining (ICCS) as previously described (Marin-Lopez, Otero-Romero et al. 2014; de la Poza, Marin-Lopez et al. 2015).

ELISPOT assays were performed with Mouse IFN gamma ELISPOT Ready-SET-Go (eBioscience), according to the method recommended by the manufacturer. A total of 5 x 10^5splenocytes were added to the well and stimulated with 10 µg/ml of recombinant NS1 protein. Plates were incubated at 37 ºC and 5% CO_2 for 18–20 h. As a positive control, PHA was used. Plates were scanned on an ImmunoSpot reader (Cellular Technology Ltd.). Specific spots were counted using the Immuno-Spot software. The threshold value to consider a positive response by ELISPOT was that the number of specific spots/well had to be at least 2 times the average values found in negative control wells of each group, and that after subtraction of background values (MS protein stimulated splenocytes). For the ICCS assay, a total of 10^6 splenocytes were stimulated with 10 µg/ml of NS1-152 peptide, concanavalin A as inespeficic stimulus (4 µg/ml), NS1-14 peptide as irrelevant peptide (10 µg/ml) or left untreated during 18 h in RPMI 1640 supplemented with 10% FCS and containing brefeldin A (5 µg/ml) to increase the accumulation of gamma interferon (IFNγ) in the responding cells. After stimulation, cells were washed, stained for the surface markers, fixed and permeabilized with PBS 1% FBS formaldehyde 4% Saponine 1% buffer and stained intracellularly using the appropriate fluorochromes. To analyze the adaptive immune responses, the following fluorochrome-conjugated antibodies were used: anti-mouse CD8-PerCP-cyanine 5.5, IFNγ-PE, from eBioescience and CD107a-(LAMP-1)-FITC from Miltenyi. Data were acquired by FACS analysis on a FACSCalibur (Becton Dickinson). Analyses of the data were performed using FlowJo software version X0.7 (Tree Star, Ashland, OR). The number of lymphocyte-gated events was 5 x 10^5.

Adoptive transfer of serum and CD8 T cells.

MVA-NS1 or MVA-wt immune CD8+T cells were isolated from the spleen of IFNAR(-/-) mice on day 10 after boost using magnetic negative CD8+ T cell selection kit (CD8a mouse+ T Cell Isolation Kit Miltenyi Biotech) and pooled. A total of 10^6 or 6 x 10^6 CD8+ T cells were transferred into the retrobulbar sinus of 8-week-old naive mice. Sera of immunized animals were collected and pooled, and 200 µl were transferred intraperitoneally. Recipient mice were challenged subcutaneously with a lethal dose of BTV-4 simultaneously with the transfer. Viral titers in blood were measured by plaque assay and clinical signs were also evaluated.

In vivo infections.

Two weeks after immunization, mice were subcutaneously inoculated with 10^2 PFUs of BTV-1 or BTV-8, 5 x10^2 PFUs of BTV-4, 10 PFUs of BTV-4M or 10^4 PFUs of BTV-16 (lethal doses). Mice were bled before each immunization and after virus challenged at 3, 5, 7, 10 and 15 d.p.i. Sera were tested for

BTV-4 neutralizing antibodies by a Virus Neutralization Test (VNT) and ELISA. Blood was collected at different times to analyzed viremia by plaque assay or measured by real-time RT-qPCR specific for BTV segment 5.

Blood measurements.

A multiparameter, Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used to determine the total and differential cell counts in blood collected into EDTA tubes.

STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism version 6.0 for Windows (GraphPad Software; San Diego, CA). Survival analysis was performed using Log-rank test. Independent-samples Student's t test was performed to compare mean responses between two groups (for viremia and Blood parameters). Wilcoxon signed rank test was performed to compare mean responses between two groups for VNTs. ELISPOT and ICCS analysis were performed using Mann-Whitney non parametric test. A value of p % 0.05 was considered significant in all cases.

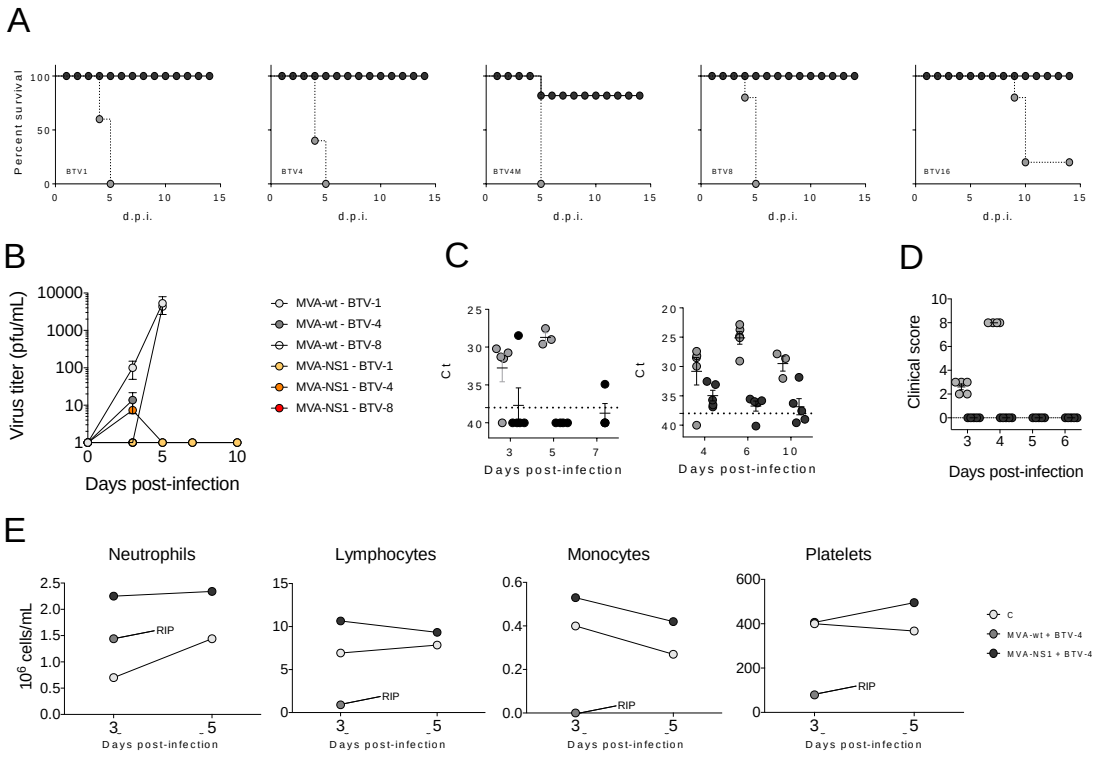


Figure 1. MVA-NS1 confers protection against multiple serotypes of BTV in IFNAR (-/-) mice.

(A) Survival rate after infection. Animals were inoculated with 10^7 PFU of MVA-NS1 or MVA-wt as negative control (non-immunized) following a prime-boost strategy. Afterwards, animals were challenged with a lethal dose of different BTV serotypes. In this and subsequent C, D and E figures, a color code was adopted to illustrate the different immunization groups: MVA-NS1 immunized mice: black circle, continued line. Non-immunized: grey circle, discontinued line. Naïve mice: white circle (just shown in E). (B) Viral titers of BTV-1, BTV-4 and BTV-8 recovered in blood of non-immunized and immunized IFNAR(-/-) mice after challenge. Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the individual values of the viral titer of each animal, and means are shown as bars. (C) Detection of BTV-4 Morocco strain (BTV-4M) (left) and BTV-16 (right) in blood of non-immunized and immunized IFNAR(-/-) mice after challenge by RT-qPCR_S5. Total RNA from blood, and the expression of mRNA of segment 5 (encoding NS1 protein) was quantified at days 3, 5 and 7 post-infection for BTV-4-M and 4, 6 and 10 post-infection for BTV-16. Results expressed as Ct and transferred to negative (neg.) according to the cut-off Ct≥38 described by Toussaint et al. (2007). (D) Post-challenge sickness score in non-immunized and immunized IFNAR(-/-) mice challenged with BTV-4. Animals were evaluated and scored for individual signs. Rough hair (absent = 0, slightly = 1, markedly = 2), activity (normal = 0, slightly reduced = 1, reduced = 2, severely reduced = 3), eye swelling (absent = 0, slightly = 1, moderate= 2, severe = 3) and temperature (normal = 0, hypothermia = 3). The final score was the addition of each individual score. The minimum score was 0 for healthy and 1–11 depending upon the severity. Animals that reached 10 points of score were euthanized. Each score represents the value of a single animal. (E) Blood parameters in non-immunized and immunized IFNAR(-/-) mice infected with BTV-4. Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used in these experiments. Neutrophils, lymphocytes, monocytes and platelets were analyzed at days 3 and 5 post-infection. Blood of non-immunized and

non-infected animals (naïve) were used for reference values. *See also the experiments presented in S3 for challenges with BTV-1, BTV-4M, BTV-8 and BTV-16.*

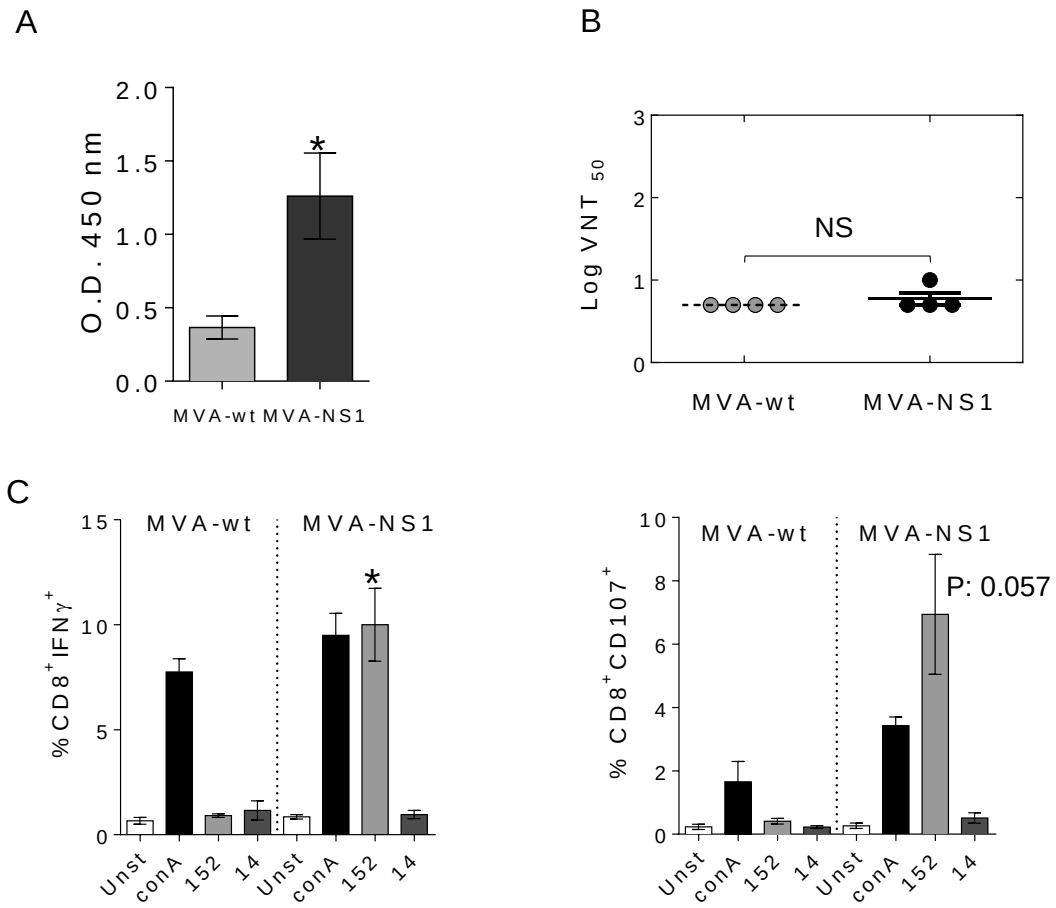


Figure 2. Immunogenicity of MVA-NS1 recombinant viral vector.

(A) Analysis of the presence of antibodies specific of NS1 in serum of non-immunized (MVA-wt) and prime-boost immunized (MVA-NS1) IFNAR(-/-) mice by ELISA. Serum of immunized mice was collected 10 days post-boost, and dilution 1:50 was analyzed by ELISA as described in Materials & Methods. (B) BTV-4 neutralizing antibody detection in non-immunized and MVA-NS1 immunized mice by VNT. Neutralization titers in sera of immunized and non-immunized animals 10 days post-boost are shown in grey circles (MVA-wt: non-immunized mice) and black circles (MVA-NS1: immunized mice). Standard deviations are shown as error bars. Asterisks indicate statistical significance calculated using the non-parametric Mann–Whitney test ($p < 0.05$). NS indicates non-significant differences. (C) Intracellular staining of IFN-γ (left) or CD107a (right), in T CD8+ cells of MVA-NS1 immunized animals. 10 days after the second immunization, spleens were harvested and the splenocytes were stimulated with NS1-152 peptide, using concanavalin A as nonspecific stimulus, NS1-14 peptide as irrelevant peptide and RPMI medium as negative control (unstimulated, *unst*). At 24 h post-stimulation, intracellular IFNγ production was analyzed in CD8+ cells and at 6 h post-stimulation, the indirect marker of cytotoxicity CD107a was also measured in CD8+ cells by flow cytometry. The results represent the average of 4 mice ± SD. Asterisks represent significant difference between samples, calculated by Mann–Whitney non-parametric test ($p < 0.05$).

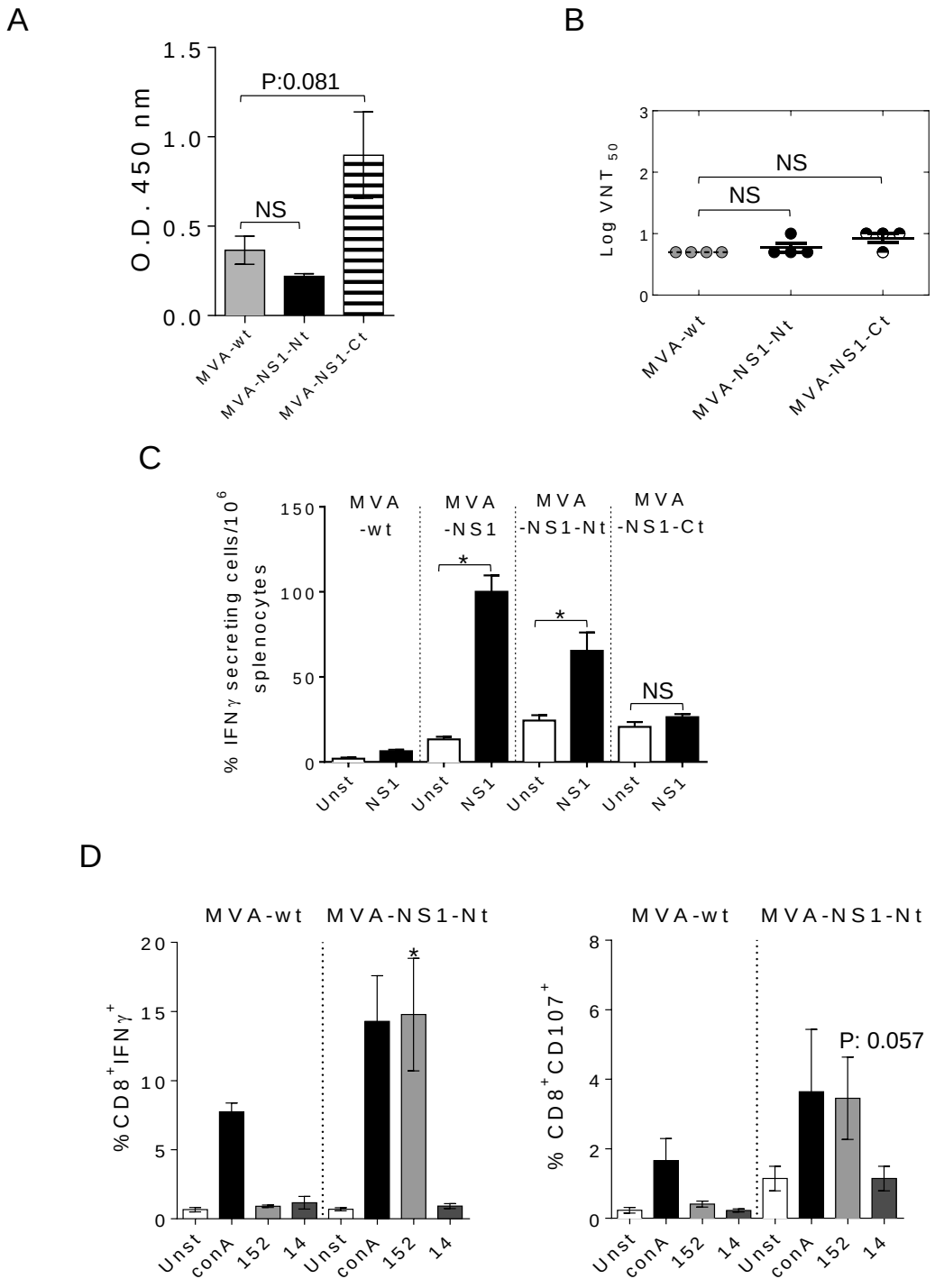


Figure 3. Immunogenicity of MVA-NS1-Nt and MVA-NS1-Ct recombinant viral vectors.

(A) Analysis of the presence of antibodies specific of NS1 in serum of non-immunized (MVA-wt) and prime-boost immunized MVA-NS1-Nt or MVA-NS1-Ct IFNAR(-/-) mice by ELISA. Serum of immunized mice was collected 10 days post-boost, and dilution 1:50 was analyzed by ELISA as described in Materials & Methods. (B) BTV-4 neutralizing antibody detection in non-immunized, MVA-NS1-Nt or

MVA-NS1-Ct immunized mice by VNT. Neutralization titers in sera of non-immunized and immunized animals 10 days post-boost are shown in grey circles (MVA-wt non-immunized mice), black circles (MVA-NS1-Nt immunized mice) and black & white circles (MVA-NS1-Ct immunized mice). Standard deviations are shown as error bars. Asterisks indicate statistical significance calculated using the non-parametric Mann–Whitney test ($p < 0.05$). NS indicates non-significant differences. (C) ELISPOT assays measuring IFNγ-secreting T cells in the spleen of non-immunized MVA-wt and immunized MVA-NS1, MVA-NS1-Nt and MVA-NS1-Ct IFNAR(-/-) mice. Splenocytes were harvested at day 10 post-boost. White (unstimulated, *unst*) and black (stimulated, *NS1*) bars represent the SFC mean number ± SD for the ELISPOT within each group. 10 μg/ml of recombinant NS1 per well were used as stimulus in each experiment. (D) Intracellular staining of IFN-γ (left) or CD107a (right), in T CD8+ cells of MVA-NS1-Nt immunized animals performed as previously describe in *Figure 2*. At 24 h post-stimulation, intracellular IFNγ production was analyzed in CD8+ cells and at 6 h post-stimulation, the indirect marker of cytotoxicity CD107a was also measured in CD8+ cells by flow cytometry. The results represent the average of 4 mice ± SD. Asterisks represent significant difference between samples, calculated by Mann–Whitney non-parametric test ($p < 0.05$).

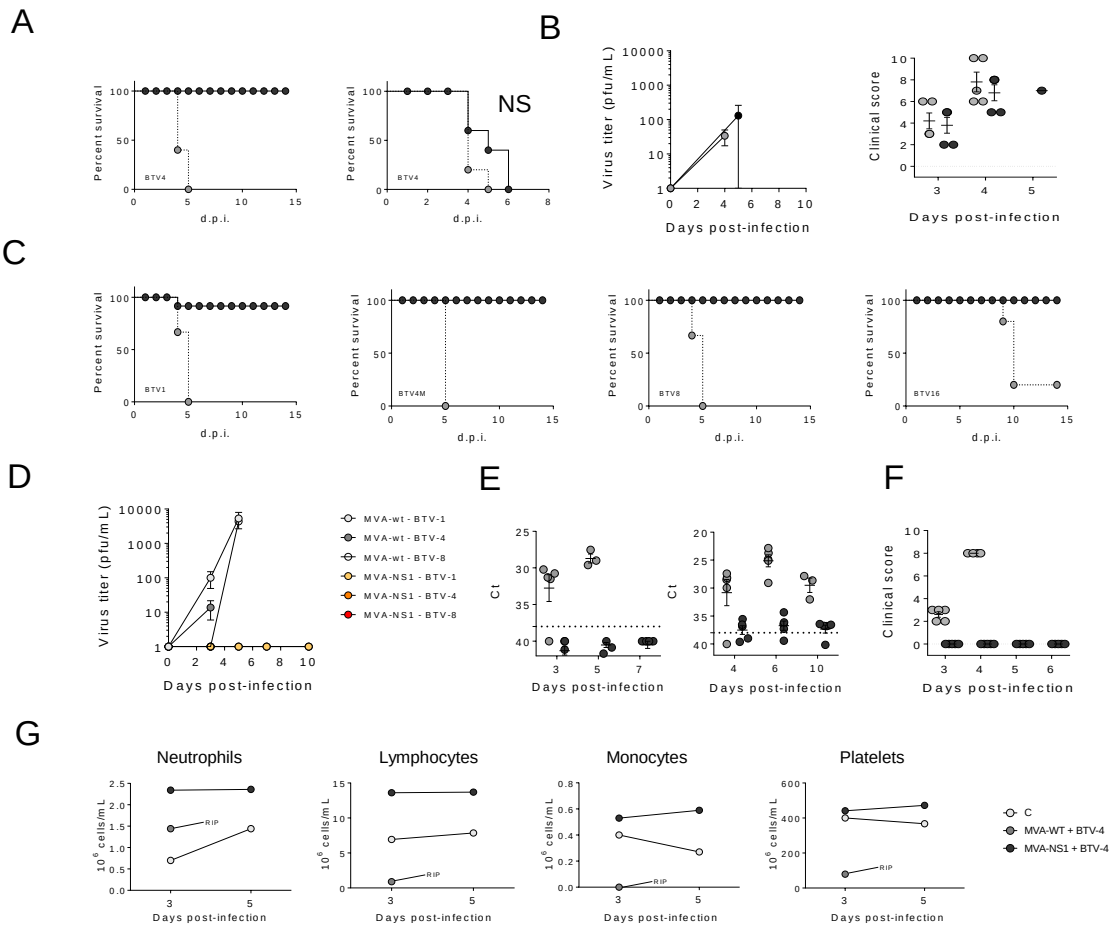


Figure 4. MVA-NS1-Nt confers protection against multiple serotypes of BTV in IFNAR (-/-) mice.

(A) Survival rate after infection. Animals were inoculated with 10^7 PFU of MVA-NS1-Nt (left), MVA-NS1-Ct (right) or MVA-wt as negative control (non-immunized) following a prime-boost strategy. Animals were challenged with a lethal dose of BTV-4 (Grey: non-immunized, black: immunized animals). NS indicates non-significant differences. (B) Viral titers of BTV-4 recovered in blood from non-immunized and MVA-NS1-Ct immunized IFNAR (-/-) mice after challenge (left) and post-challenge sickness score (right). Non-immunized animals: grey circles; immunized animals: black circles. (C) Survival rate after infection. Non-immunized animals and MVA-NS1-Nt immunized animals were challenged against BTV-1, BTV-4M, BTV-8 and BTV-16. Non-immunized animals: grey circles; immunized animals: black circles. In this and subsequent E and F figures, the same color code was adopted to illustrate the different immunization groups. (D) Viral titers of BTV-1, BTV-4 and BTV-8 recovered in blood of non-immunized and MVA-NS1-Nt immunized IFNAR (-/-) mice after challenge. Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the individual values of the viral titer of each animal, and means are shown as bars. (E) Detection of BTV-4 Morocco strain (BTV-4M) (left) and BTV-16 (right) in blood of non-immunized and immunized IFNAR(-/-) mice after challenge by RT-qPCR_S5. Total RNA from blood, and the expression of mRNA of segment 5 (encoding NS1 protein) was quantified at days 3, 5 and 7 post-infection. Results expressed as Ct and transferred to negative (neg.) according to the cut-off Ct≥38 described by Toussaint et al. (2007). (F) Post-challenge sickness score in non-immunized (blue triangle) and immunized (red square) IFNAR(-/-) mice challenged with BTV-4. Animals were evaluated and scored for individual signs as described in Figure 1. (G) Blood parameters in non-immunized and MVA-NS1-

Nt immunized IFNAR(-/-) mice infected with BTV-4. Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used in these experiments. Neutrophils, lymphocytes, monocytes and platelets were analyzed at days 3 and 5 post-infection. Blood of non-immunized and non-infected animals (naïve) were used for reference values. *See also the experiments presented in S1 for challenges with BTV-1, BTV-4M, BTV-8 and BTV-16.*

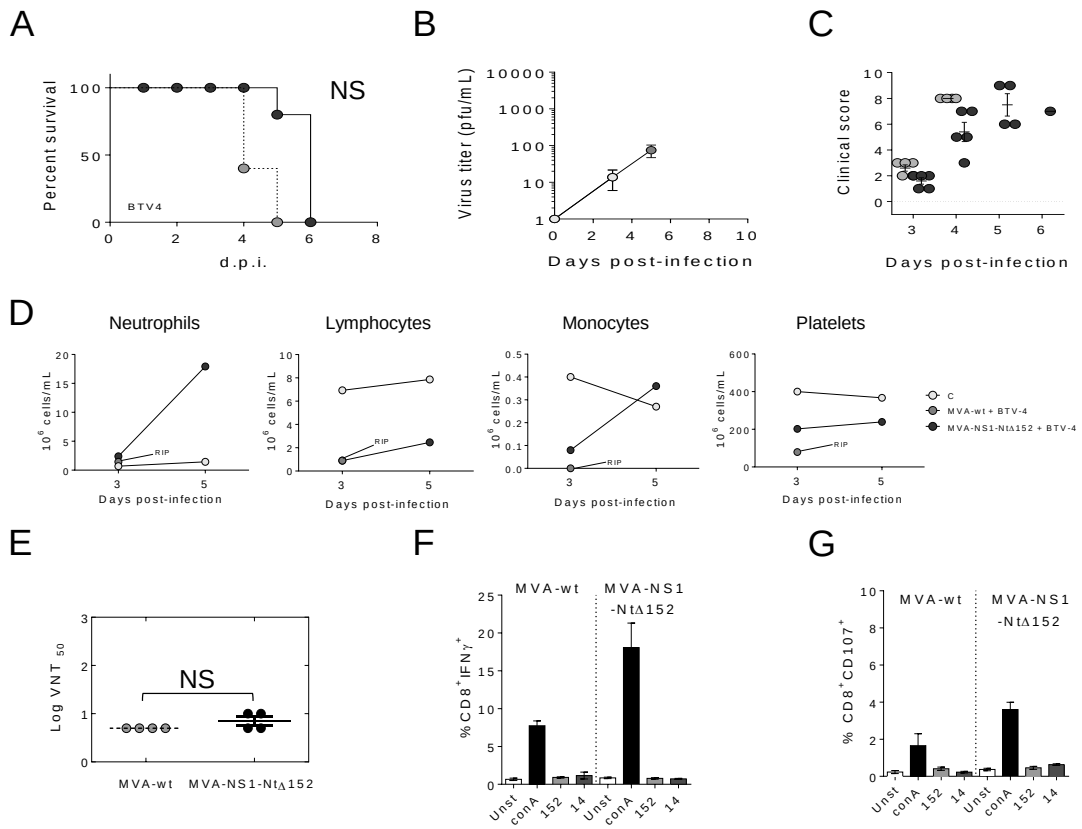


Figure 5. MVA-NS1-NtΔ152 losses it protection capacity against BTV-4 in IFNAR (-/-) mice.

(A) Survival rate after infection. Animals were inoculated with 10^7 PFU of MVA-NS1-NtΔ152 or MVA-wt as negative control (non-immunized) following a prime-boost strategy and challenged with a lethal dose of BTV-4. In this and subsequent B, C, D an E figures, a color code was adopted to illustrate the different immunization treatment: grey circles (MVA-wt non-immunized), black circles (MVA-NS1-NtΔ152) and white circles (naïve animals). (B) Viral titers of BTV-4 recovered in blood of MVA-wt non-immunized and MVA-NS1-NtΔ152 immunized IFNAR (-/-) mice after challenge. Each point represents the individual values of the viral titer of each animal, and means are shown as bars. (C) Post-challenge sickness score in non-immunized and MVA-NS1-NtΔ152 immunized IFNAR(-/-) mice challenged with BTV-4. Animals were evaluated and scored for individual signs. The minimum score was 0 for healthy and 1–11 depending upon the severity. Each score represents the value of a single animal. (D) Blood parameters in non-immunized and MVA-NS1-NtΔ152 immunized IFNAR(-/-) mice infected with BTV -4. Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used in these experiments. Neutrophils, lymphocytes, monocytes and platelets were analyzed at days 3 and 5 post-infection. Blood of non-infected and non-immunized animals (naïve) were used for reference values. (E) BTV-4 neutralizing antibody detection in non-immunized and MVA-NS1-NtΔ152 immunized mice by VNT. Neutralization titers in sera of immunized and non-immunized animals 10 days post-boost are shown. Standard deviations are shown as error bars. Asterisks indicate statistical significance calculated using the non-parametric Mann–Whitney test ($p < 0.05$). (F) Intracellular staining of IFN-γ (left) or CD107a (right), in T CD8+ cells of MVA-NS1-NtΔ152 immunized animals. 10 days after the second immunization, spleens were harvested and the splenocytes were stimulated with NS1-152 peptide, using concanavalin A as nonspecific stimulus, NS1-14 peptide as irrelevant

peptide and RPMI medium as negative control (unstimulated, *unst*). At 24 h post-stimulation, intracellular IFNγ production was analyzed in CD8+ cells and at 6 h post-stimulation, the indirect marker of cytotoxicity CD107a was also measured in CD8+ cells by flow citometry. The results represent the average of 4 mice ± SD. NS indicates non-significant differences.

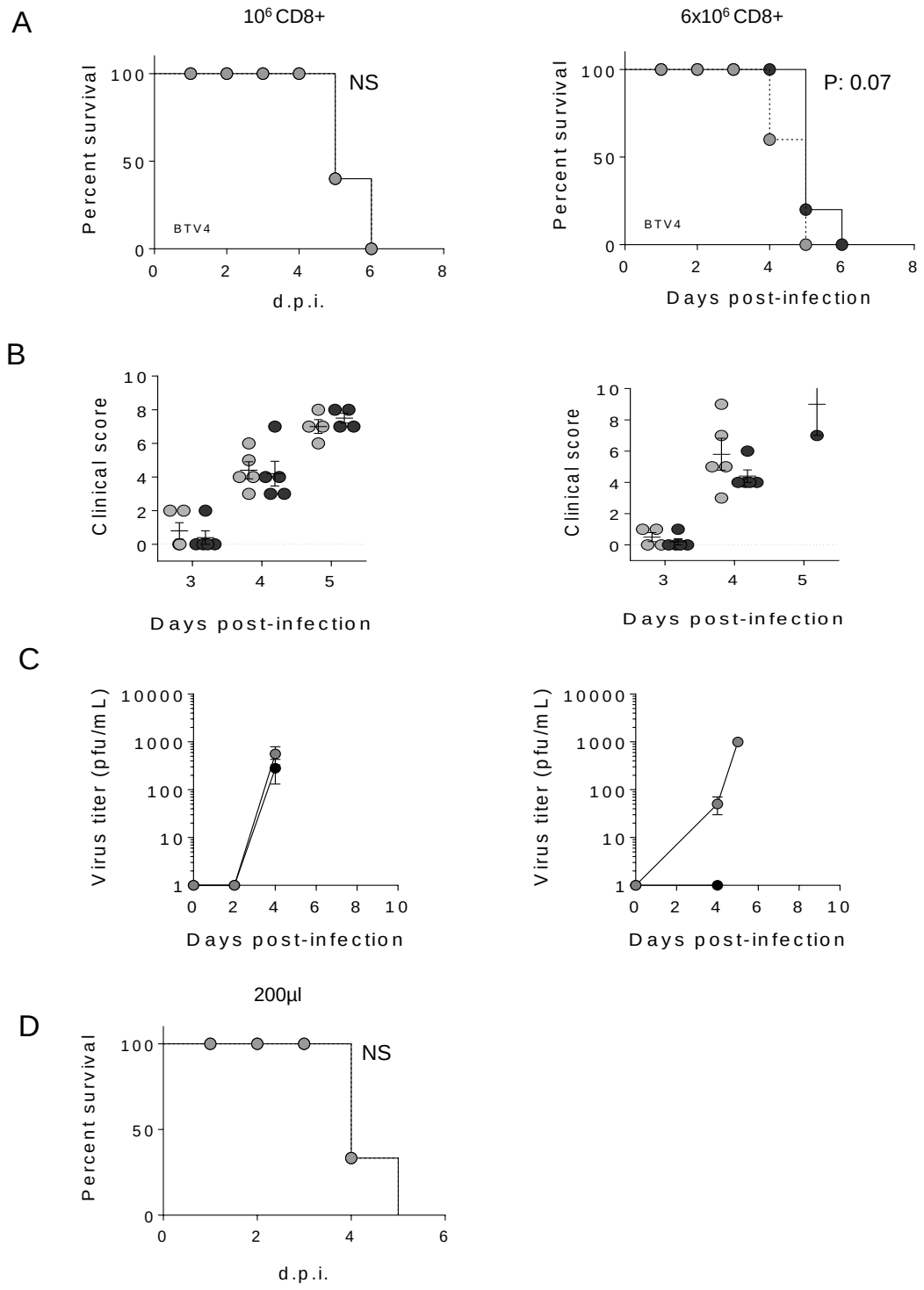


Figure 6. Protective role of CD8+ T cells from MVA-NS1 immunized mice against BTV infection.

(A) Five animals were inoculated with 10^7 PFU of MVA-NS1 or MVA-wt as negative control (non-immunized) following a prime-boost strategy. After 10 days post-boost, animals were euthanized, and spleens were collected. CD8+ cells were enriched from the splenocytes by negative selection as described in Materials & Methods and pooled. Eight-week-old naïve IFNAR(-/-) mice were transferred retroorbitally with 10^6 CD8+ T cells or $6x10^6$ CD8+ T cells from non-immunized (grey circles) or immunized (black circles) animals and challenged with a lethal dose of BTV-4 subcutaneously.

Survival was monitored during the experiment in both groups (left, 10^6 CD8+ T cells transfer; right, $6x10^6$ CD8+ T cells). Subsequent B, C and D figures maintain the same color code. (B) Post-challenge sickness score in animals transferred with 10^6 CD8+ T cells from non-immunized (grey circles) and immunized (black circles) IFNAR(-/-) mice, and then challenged with BTV-4 (left) or transferred with $6x10^6$ CD8+ T cells (right) . Animals were evaluated and scored as described in Figure 1. Each score represents the value of a single animal. (C) Viral titers BTV-4 recovered in blood of transferred animals after challenge. 10^6 CD8+ T cells transferred animals (left) and $6x10^6$ CD8+ T cells transferred animals (right). Virus was extracted from blood and determined as described in Materials and Methods. Each point represents the individual values of the viral titer of each animal, and means are shown as bars. (D) Passive serum transfer. 200 µl of pooled sera from MVA-NS1 immunized or non-immunized animals were transfer intraperitoneally into naïve IFNAR(-/-) mice and challenged with a lethal dose of BTV-4 subcutaneously. Survival was analyzed during the experiment. Log-rank (Mantel-Cox) test was used to compare groups. NS indicates non-significant differences.

A

NS1 primers	Sequence 5'-3'
NS1 SmaI Fw 1	CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC
NS1 SmaI Rs 786	CGCCCGGGCTAACCTGTCGGAACCTTTCCAAAAAG
NS1 SmaI Fw 811	CGGAATTCATGGAATTTCCATTACATCAGATGATGC
NS1 SmaI Rs 1628 (1659)	CGCCCGGGCTAATACTCCATCCACATCTG
NS1 Δ152 Fw	AATCAAAGAATCAAGATATCGCCAAATTGCATACTATTTC
NS1 Δ152 Rs	GATATCTTGATTCTTTGATTGGCACATAGATGTAAGGCAT

B

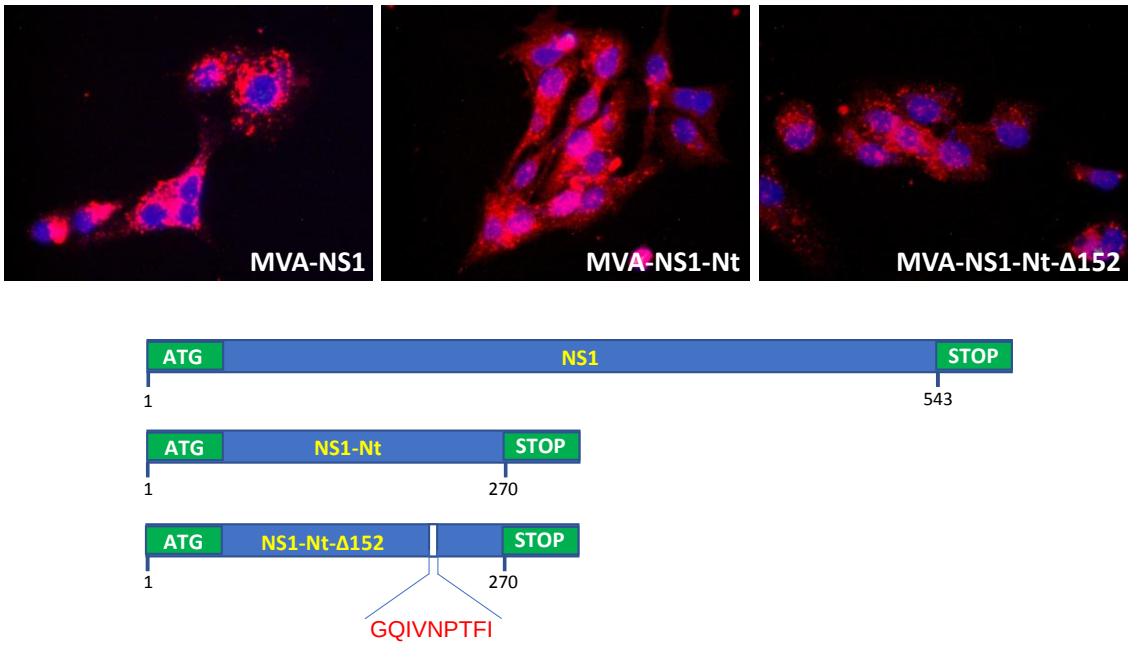
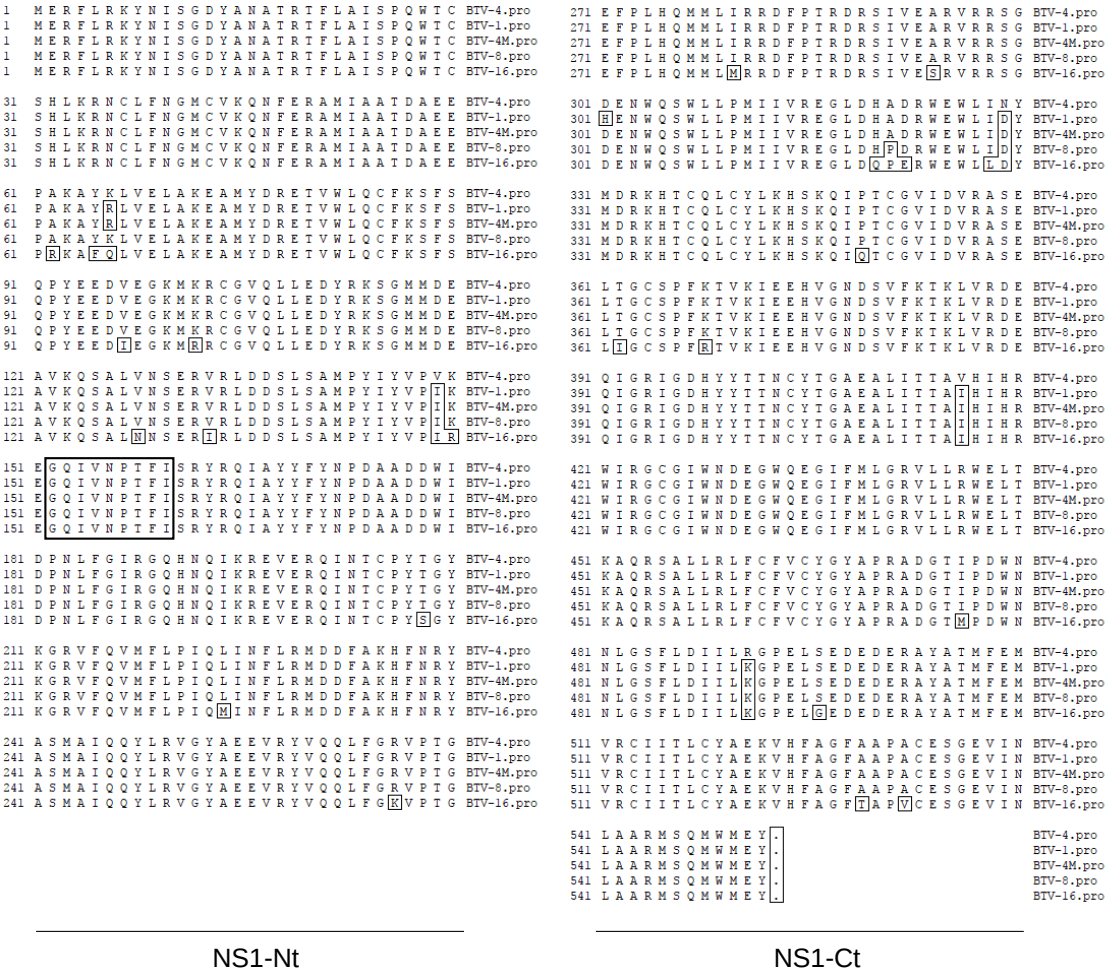


Figure S1. Table of primers and immunofluorescence of rMVAs.

(A)Table of primers used to generate NS1-Ct, NS1-Nt and NS1-NtΔ152. (B)Indirect immunofluorescence of DF-1 cells infected with MVA-NS1, MVA-NS1-Nt and MVA-NS1-NtΔ152 at 24 h.p.i. using a mouse polyclonal serum specific of BTV-4. Alexa Fluor goat anti-mouse 594 (Life Technologies) was used as secondary antibody. Scheme of the NS1, NS1-Nt and NS1-NtΔ152 constructions, with the deletion of p152 in NS1-NtΔ152.

A



B

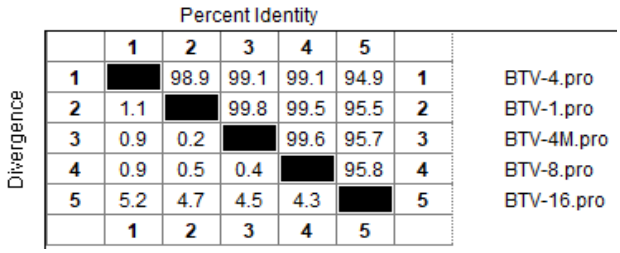


Figure S2. Sequence alignment and percentage of identity among NS1 sequences.

(A) NS1 sequence alignment of BTV-1, BTV-4, BTV-4M, BTV-8 and BTV-16. The CD8 T cell epitope 152 was boxed in the figure. (B) Percentage of identity among NS1 sequences of BTV-1, BTV-4, BTV-4M, BTV-8 and BTV-16.

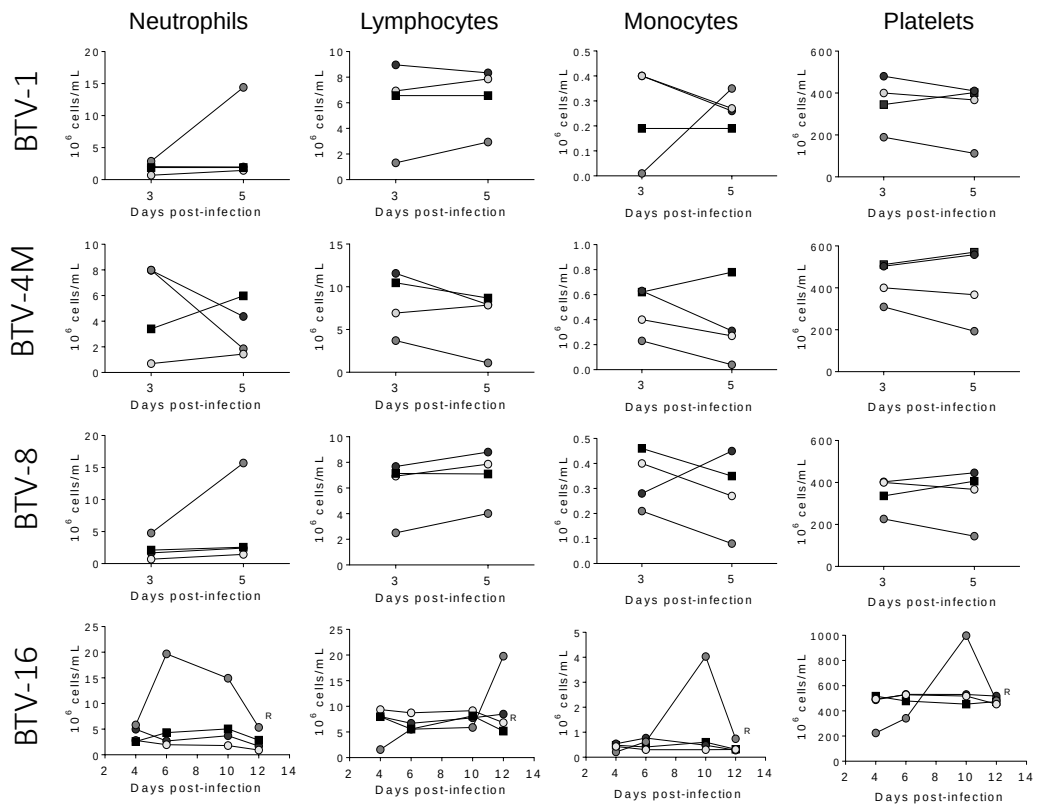


Figure S3. Blood parameters in non-immunized and immunized IFNAR(-/-) mice infected with BTV-1, BTV-4M, BTV-8 and BTV-16.

Autohematology Analyzer (BC-5300 Vet, Mindray, China) was used in these experiments. Neutrophils, lymphocytes, monocytes and platelets were analyzed at days 3 and 5 post-infection for BTV-1, BTV-4M, BTV-8 and 4,6,10 and 12 for BTV-16. Blood of non-immunized and non-infected animals (naïve, white circles) were used for reference values. Non-immunized and infected animals (clear grey circles), MVA-NS1 immunized and infected animals (dark grey circles) and MVA-NS1-Nt immunized and infected animals (black squares).

DISCUSIÓN

La propagación mundial y la incidencia de las enfermedades arbovirales (que son transmitidas por mosquitos, moscas o garrapatas) ha aumentado en las últimas décadas, con un impacto significativo en la salud animal y humana, afectando a la seguridad alimentaria. Estos cambios se han relacionado con el cambio climático (que influye en la actividad y distribución de los vectores artrópodos), así como con el aumento del comercio y la globalización (la introducción de patógenos exóticos en nuevas zonas). La enfermedad de la lengua azul representa una amenaza importante para la salud del ganado y la producción de alimentos en Europa y en los países vecinos. La continua llegada de nuevas cepas «exóticas» procedentes de regiones vecinas sugiere que es muy probable que las incursiones de BTV (y posiblemente también de otros arbovirus relacionados) continúen en Europa en el futuro. Aunque las vacunas convencionales han controlado o limitado la propagación de la BTV en el pasado, no pueden abordar la necesidad de protección cruzada entre todos los serotipos de BTV. Además, estas vacunas no permiten distinguir entre animales infectados y vacunados (estrategia DIVA). Por lo tanto, un objetivo importante en el campo del desarrollo de vacunas frente a BTV es el desarrollo de vacunas universales que generen una protección eficaz frente a los múltiples serotipos de este virus.

1. CARACTERIZACIÓN PATOLÓGICA DE LA INFECCIÓN POR BTV-4 EN EL MODELO DE RATÓN IFNAR(-/-).

Los rumiantes son los hospedadores naturales del virus de la lengua azul y por ende, el tipo de animal preferido para llevar a cabo ensayos de infección experimentales. Sin embargo, los ensayos de infección en rumiantes presentan una serie de desventajas como son su elevado coste, el tiempo que ha de invertirse en su desarrollo y el número limitado de animales que se pueden utilizar (Coetzee et al., 2014). El uso de ratones IFNAR(-/-) permite en gran medida minimizar dichas limitaciones debido a su menor coste económico y a la facilidad para trabajar con ellos. Otra ventaja muy importante que confiere el uso de este modelo animal es la amplia disponibilidad de reactivos para llevar a cabo estudios de respuesta inmune frente al virus. El ratón IFNAR(-/-) se ha utilizado como modelo animal en el estudio de la transmisión, patogénesis y virulencia de BTV, así como en la evaluación de la eficacia de vacunas frente a BTV tanto inactivadas como recombinantes marcadoras (Calvo-Pinilla et al.,

2012; Caporale et al., 2014; Legisa et al., 2015; Ma et al., 2012; Marin-Lopez et al., 2014; Ortego, de la Poza, and Marin-Lopez, 2014; Ratinier et al., 2011; Rojas et al., 2011). La extrapolación de los resultados obtenidos en el modelo de ratón al hospedador natural rumiante ha de realizarse con cautela, debido a las diferencias en la biología de estos animales. La susceptibilidad de este modelo a la infección por BTV-1, BTV-4 y BTV-8 se ha caracterizado previamente en nuestro laboratorio (Calvo-Pinilla, Nieto, and Ortego, 2010; Calvo-Pinilla et al., 2009a; Ortego, de la Poza, and Marin-Lopez, 2014). Estos tres virus habían sido aislados en huevos embrionados y adaptados posteriormente a crecer en cultivo de células BHK o Vero. En este trabajo se ha utilizado la cepa BTV-4M (MOR2009/09), aislada a partir de sangre de ovejas infectadas en la línea celular KC derivada de *Culicoides*, sin ser pasada por líneas celulares de mamífero. De esta forma se ha pretendido analizar la patogénesis generada por un virus que mimetiza mejor lo que ocurre en la naturaleza, evitando los posibles artefactos debidos a la adaptación del virus a crecer en células de mamífero. La clínica de la enfermedad producida por BTV-4M en el ratón cursó con viremia y altos títulos virales en pulmón y tejidos linfoides como bazo y timo. A esto se le asoció neutrofilia, linfopenia, trombocitopenia y una bajada drástica en el nivel de monocitos en sangre. Estos resultados reproducen aquellos observados en infecciones experimentales en el hospedador natural, como es el caso de un ensayo en el que se infectaron experimentalmente ovejas con BTV-23 y otro trabajo en el que se infectaron ciervos de cola blanca (*Odocoileus virginianus*) con BTV-17, donde se observó un marcado descenso en el nivel de linfocitos y plaquetas así como un aumento en el nivel de neutrófilos (Howerth and Tyler, 1988; Maclachlan et al., 2009; McColl and Gould, 1994). Estos cambios en los parámetros hematológicos observados en ratones IFNAR(-/-) infectados con BTV correlacionan con la presencia de viremia y son detectados incluso antes de la aparición de signos clínicos en los animales. Por ello, el estudio hematológico durante la infección de BTV supone una herramienta muy valiosa y no invasiva en el estudio de eficacia de vacunas tanto en el modelo de ratón como en el huésped natural.

Por tinción con hematoxilina-eosina e inmunomarcaje de CD3 y CD79 de secciones de bazo y timo de ratones IFNAR(-/-) inoculados con BTV-4M se observó depleción linfoide e infiltración de neutrófilos en estos tejidos. Esta reducción de linfocitos B y T en tejidos linfoides se ha descrito en infecciones en oveja con diferentes serotipos de BTV. Uno de los mecanismos que parece estar involucrado en la depleción linfoide es la apoptosis (Umeshappa et al., 2010). Además, la replicación de BTV en los linfocitos, macrófagos y en las células dendríticas puede conllevar un daño celular (Darpel et al., 2012). Dado que hemos observado marcaje específico para BTV en linfonodos, bazo y timo de ratones IFNAR(-/-) infectados (Calvo-Pinilla, Nieto, and

Ortego, 2010), asi como un fuerte incremento de la actividad caspasa-3 en dichos tejidos, indica que ambos mecanismos, tanto apoptosis como la propia replicación del virus, pueden estar implicados en la depleción linfoide observada en el modelo de ratón y en rumiantes. Por otro lado, hemos observado un aumento de monocitos/macrófagos en bazo y timo, y en menor medida, en pulmón, por inmunomarcaje con un anticuerpo específico de MAC387, al igual que se ha descrito en rumiantes infectados con BTV (Lee et al., 2011; Sanchez-Cordon et al., 2010), cuya función parece ser la de capturar, internalizar y procesar los antígenos del virus.

Estudios previos, tanto *in vivo* como *in vitro* han puesto de manifiesto la secreción de múltiples citoquinas proinflamatorias como IFN-α, IFN-γ, IL-12, TNF, IL-1ß e IL-8 por parte de los leucocitos mononucleares infectados (células dendríticas, macrófagos, monocitos y algunos linfocitos) (Channappanavar et al., 2012; Drew et al., 2010b; Hemati et al., 2009; Schwartz-Cornil et al., 2008; Umeshappa et al., 2011). La inducción de una respuesta proinflamatoria antiviral es esencial para controlar la replicación del virus y para activar una respuesta adaptativa adecuada. Aunque estas respuestas se requieren para el aclaramiento de la infección pueden acarrear daño en los tejidos contribuyendo a la patogénesis de la enfermedad (Reiss and Komatsu, 1998). El daño en el endotelio de rumiantes infectados con BTV puede ser debido a un efecto directo de la infección por BTV en las células endoteliales o debido a una respuesta frente a los mediadores inflamatorios liberados por las células endoteliales infectadas y, muy posiblemente, por otros tipos celulares como monocitos y macrófagos (Channappanavar et al., 2012; Drew et al., 2010b; Hemati et al., 2009; Sanchez-Cordon et al., 2013). Además, infecciones *in vitro* de células periféricas mononucleares sanguíneas (PBMCs) de varias especies de rumiantes (bovino, ovino y caprino) con BTV desencadenan la producción de citoquinas inflamatorias como IL-1,6,8 y 10, IFN-γ, TNF e iNOS (Dhanasekaran et al., 2013; Drew et al., 2010b), aunque estos estudios se llevaron a cabo siguiendo diferentes diseños experimentales y serotipos de BTV, dificultando la correlación de resultados entre especies.

En este trabajo analizamos con detalle si la presencia de macrófagos en bazo, timo y pulmón de ratones IFNAR(-/-) infectados con BTV correlaciona con la activación de una respuesta proinflamatoria en estos tejidos y observamos un incremento en la transcripción de los genes que codifican IL-1ß, IL-6, IL-12p40, IFN-γ y TNF que correlaciona con la elevada presencia de macrófagos tanto en bazo como en timo y con una marcada alteración histopatológica en estos tejidos diana. Además de la inducción de citoquinas proinflamatorias detectadas tanto en suero como en los tejidos de los rumiantes infectados por BTV, se ha

observado también un aumento en la transcripción de iNOS en PBMCs y órganos linfoides secundarios (Umeshappa et al., 2012). Estudios *in vitro* han evidenciado que tanto citoquinas como otras sustancias vasoactivas producidas por macrófagos contribuyen a la aparición de daño vascular en rumiantes infectados (Drew et al., 2010b). El modelo de ratón IFNAR(-/-) reprodujo *in vivo* el incremento en la expresión de iNOS en los tejidos infectdos con BTV-4M. El número de células positivas (monocitos/macrófagos y neutrófilos) para el marcaje con un anticuerpo específico de iNOS en ratones infectados con BTV-4M aumentó ligeramente en pulmón y significativamente en bazo y timo. El óxido nítrico tiene un potente efecto antiviral a la hora de combatir la infección junto con las citoquinas. Sin embargo, la producción excesiva de iNOS, y como consecuencia el aumento excesivo de los niveles de óxido nítrico, durante la infección viral puede conllevar efectos negativos, promoviendo, junto con otros oxidantes, una excesiva respuesta inflamatoria y apoptosis, como se ha descrito en la infección por el virus influenza (Burggraaf et al., 2011). Como ya se ha descrito en otros trabajos donde estudian la patogénesis producida por virus que causan fiebres hemorrágicas (Basu and Chaturvedi, 2008; Bray, 2005; Marty, Jahrling, and Geisbert, 2006), la sobreexpresión de iNOS y citoquinas proinflamatorias en tejidos infectados pueden contribuir a la patología de la enfermedad, causando vasodilatación, incremento de la adhesión leucocitaria y formación de trombos, además de un aumento en la permeabilidiad vascular del endotelio, el cual conduce a la aparición de edemas (Drew et al., 2010b; Lentsch and Ward, 2000). Por tanto, la inducción observada de citoquinas proinflamatorias e iNOS en tejidos de ratones IFNAR(-/-) infectados con BTV podrían explicar al menos en parte la patología hemorrágica observada en rumiantes infectados con este virus.

BTV induce apoptosis tanto en cultivo celular como en los órganos diana donde replica. Una hipótesis es que los mecanismos de apoptosis desempeñan un papel clave en la patogénesis producida por la infección por BTV (Mortola, Noad, and Roy, 2004; Nagaleekar et al., 2007; Stewart and Roy, 2010). En este trabajo observamos que el bazo de los ratones infectados con BTV mostró debris apoptótico y una presencia alta de actividad caspasa-3, reproduciendo el efecto apoptótico observado en las ovejas infectadas por BTV. La actividad caspasa-3 es una importante molécula proapoptótica, actuando como efector final de todas las rutas de apoptosis, con lo que se considera como un buen marcador de apoptosis (Hengartner, 2000). Es llamativo que el número de células positivas para el marcador de caspasa-3 no es lo suficientemente alto como para explicar la necrosis masiva y la depleción linfoide que tiene lugar en el bazo y timo de los ratones infectados. Esto posiblemente sea debido a que no es posible detectar la actividad caspasa-3 en todas las células apoptóticas (Liang, Yan, and Schor,

2001) y a que también se dan procesos de necrosis en estos tejidos, probablemente debido a un incremento del ambiente oxidativo. El papel de la actividad caspasa-3 en la apoptosis inducida por la infección por BTV se ha confirmado en líneas celulares (Mortola, Noad, and Roy, 2004) y en ovejas infectadas por BTV-23, donde se observó apoptosis en PBMCs y bazo y aumento de los mRNAs correspondientes a caspasa-3 (Umeshappa et al., 2010). Hay estudios que sugieren que el IFNα podría ser un mediador de la apoptosis, activando la liberación de citoquinas proinflamatorias y mediando la producción de óxido nítrico en células endoteliales y macrófagos activados (Umeshappa et al., 2010). La inducción de apoptosis en los ratones IFNAR(-/-) infectados con BTV, en los cuales la ruta de señalización del IFNα está bloqueada, indica que la infección producida por BTV puede desencadenar procesos de apoptosis a través de una vía independiente de IFNα.

Los rumiantes son actualmente el mejor modelo animal para estudiar la infección por BTV. Cualquier candidato vacunal o tratamiento antiviral tiene que haber tenido éxito en rumiantes antes de pasar a ensayos clínicos. Sin embargo, teniendo en cuenta consideraciones éticas, prácticas y económicas, un modelo murino que reproduzca los aspectos patológicos de la infección es una valiosa herramienta para ensayar la eficacia de potenciales vacunas y para estudiar la transmisión, patogénesis y virulencia de BTV.

2. DESARROLLO DE ESTRATEGIAS COMBINADAS DE VACUNACIÓN MULTISEROTIPO FRENTE A BTV BASADAS EN MICROESFERAS DE LA PROTEÍNA muNS-MI DE REOVIRUS AVIAR Y EL VECTOR VIRAL MVA QUE EXPRESEN LOS ANTÍGENOS VP2, VP7 Y NS1.

La vacunación es uno de los métodos más efectivos para controlar la expansión de BTV. Las vacunas comerciales, basadas en vacunas inactivadas por métodos químicos, protegen frente a la infección por BTV, pero son específicas de serotipo y no permiten la diferenciación entre animales vacunados e infectados. Como consecuencia, en la actualidad se están desarrollando nuevas vacunas más seguras y eficaces y que permitan la protección frente a múltiples serotipos (Boone et al., 2007; Calvo-Pinilla et al., 2012; Celma et al., 2013; Franceschi et al., 2011; Jabbar et al., 2013; Noad and Roy, 2009; Perrin et al., 2007; Roy and Noad, 2009). Uno de los mayores esfuerzos se concentra en generar vacunas recombinantes de tipo subunidad. En los últimos años se están utilizando biomateriales como plataforma vacunal ya que permiten controlar las propiedades físico-químicas del material y también permiten la adición de antígenos, adyuvantes o moléculas diana para que sean reconocidos por el sistema inmune. Los adyuvantes convencionales como el aluminio, mejoran la respuesta

inmune de tipo humoral, pero los biomateriales particulados no solo mejoran este tipo de respuesta, sino también la respuesta de tipo celular. Además se ha comprobado, que repetidas inoculaciones de adyuvantes basados en sales de aluminio conducen a la aparición del síndrome autoinmune/autoinflamatorio, conocido como *ASIA*, el cual puede evitarse usando vacunas basadas en biomateriales. En este trabajo hemos generado una nueva vacuna de tipo subunidad frente a BTV basada en microesferas constituidas por una región de la proteína muNS del reovirus aviar que incorpora las proteínas VP2, VP7 o NS1 de BTV-4, la cual, además de ser eficaz frente a la infección, es segura y permite aplicar la estrategia DIVA.

Las vacunas frente a BTV basadas en proteínas se han formulado tradicionalmente como proteínas individuales o combinando varias proteínas en un cóctel. La vacuna subunidad más exitosa frente a BTV se basa en la coexpresión de las cuatro principales proteínas estructurales (VP2, VP5, VP3 y VP7) lo cual conduce a la formación de *virus like particles* (VLPs) (Roy, 1990; Roy, 1992; Roy et al., 1994; Roy, French, and Erasmus, 1992). Las VLPs son inmunógenops particulados que mantienen la estructura de las proteínas en su correcta conformación, confiriendo una buena respuesta inmune, pero su producción a gran escala es costosa, con lo que su aplicación en la industria ganadera es difícil. Además, la expresión de varias proteínas al mismo tiempo para conseguir la formación de estas VLPs ha ser estar estrictamente controlada, para que haya una proporción adecuada de todos sus componentes. A estas dificultades hay que añadirle la dificultad para adaptar la purificación de VLPs a nivel industrial. Por el contrario, las microesferas basadas en la proteína muNS de ARV son fáciles de producir, purificar y muy estables. Este método de microesferas que incorporan antígenos es un método muy eficaz para dar una respuesta rápida frente a brotes de distintos serotipos en el caso de BTV o incluso de distintos virus. En cuanto a BTV, solo habría que generar las microesferas que incorporasen los antígenos que son diferentes entre serotipos. Por otra parte, hemos demostrado que es posible incorporar distintas proteínas sobre una misma microesfera, observándose interacciones complejas entre ellas (Brandariz-Nunez et al., 2010; Brandariz-Nunez et al., 2011). En trabajos anteriores de nuestro laboratorio hemos desarrolllado vacunas marcadoras y las hemos ensayado frente a diferentes serotipos de BTV, siguiendo una estrategia de vacunación *prime-boost* heteróloga, utilizando vscunas DNA y el vector viral MVA que expresan las proteínas VP2, VP7 y NS1 de BTV-4 (Calvo-Pinilla et al., 2012). Ratones IFNAR(-/-) inoculados siguiendo esta estrategia mostraron niveles significativos de anticuerpos neutralizantes frente a BTV-4 y una fuerte activación de la respuesta celular T CD8+ frente a estas tres proteínas. Esta combinación de vacunas que expresaban los tres antígenos (VP2, VP7 y NS1) confirió una protección total frente al desafío homólogo con BTV-4.

A la vista de estos resultados nos centramos en estos tres antígenos para el diseño de las microesferas. La presentación de VP2, VP7 y NS1 en las microesferas de muNS (MS-VP2, MS-VP7 y MS-NS1) y sin adyuvante protegieron a los ratones inmunizados con estas construcciones frente al desafío homólogo con una dosis letal del serotipo 4 de BTV, en ausencia de viremia durante todo el experimento. Por el contrario, ratones inmunizados con las proteínas recombinantes VP2, VP7 y NS1 producidas mediante el sistema de baculovirus sin emplear adyuvantes no se protegieron frente a la infección de BTV-4, detectándose virus infectivo en sangre. Tradicionalmente se ha asociado una buena inducción de anticuerpos neutralizantes con protección frente a la inmfección por BTV. La inmunización con las microesferas aumentó los niveles de anticuerpos neutralizantes frente a BTV-4, alcanzando unos valores similares a los obtenidos en los animales inmunizados con DNA/rMVA. Por el contrario, la inmunización con las proteínas recombinantes indujo una actividad neutralizante escasa. Estudios previos de inmunización en ratones IFNAR(-/-) en los que se inocularon VP2, VP5 y VP7 adyuvantado con Montanide ISA-50V mostraron una fuerte inducción de anticuerpos neutralizantes (Calvo-Pinilla et al., 2014), aunque siempre inferiores a los observados en la inmunización con microesferas. Todos estos datos confirman que la administración de los antígenos VP2, VP7 y NS1 de BTV-4 vehiculizados a través de las microesferas de munNS-Mi de ARV confiere una potente actividad neutralizante así como protección frente a BTV-4. Estudios sobre la interacción de BTV con el sistema inmune del hospedador mostraron que los anticuerpos neutralizantes (Jeggo, Wardley, and Taylor, 1984b) y la respuesta celular T (Jeggo and Wardley, 1982; Takamatsu and Jeggo, 1989) tienen una función importante en la inmunidad protectora frente a BTV (Maclachlan et al., 2014). La administración de microesferas en el ratón IFNAR(-/-) produce una alta inmunogenicidad, induciendo una fuerte respuesta celular T CD4+, resultados que también se observaron en ratones *wild type* con el mismo background genético (129/sv).

Los antígenos particulados exógenos, una vez son endocitados, son cargados en el complejo mayor de histocompatibilidad (MHC) de clase II, y presentados a las células CD4+ (Demento et al., 2011). La inmunización con microesferas favorece principalmente la activación de la respuesta T CD4+ en el ratón, aunque también se detectó una leve activación de la respuesta T CD8+. Se ha descrito que esta última es una vía alternativa por la que los antígenos particulados pueden escapar del endosoma y ser procesados en el citoplasma, vía proteosoma, y presentados en MHC de clase I a las células CD8+ (Demento et al., 2011).

Otro punto a destacar ha sido la protección cruzada parcial frente al desafío con BTV-1 en ausencia de anticuerpos neutralizantes específicos frente a este serotipo. La importancia de incluir la proteína NS1 en la composición de la vacuna a la hora de inducir protección cruzada

en el modelo de ratón (Calvo-Pinilla et al., 2012) se ha confirmado en los hospedadores naturales (ganado ovino y bovino) (Anderson et al., 2013; Rojas et al., 2014). La posibilidad de incluir la proteína no estructural NS1 es una ventaja adicional de las microesferas de ARV en comparación con las VLPs, donde solo se pueden incluir proteínas estructurales. Cuando NS1 se incluye en la composición vacunal, junto con VP2 y VP7 del serotipo 4, la estrategia *prime-boost* DNA/rMVA confirió protección cruzada total frente a los desafíos con dosis letales de los serotipos heterólogos BTV-1 y BTV-8, en ausencia de anticuerpos neutralizantes específicos frente a estos dos serotipos. Además se logró una potente estimulación de la respuesta CD8+ frente a VP2, VP7 y NS1, lo que sugiere que la respuesta de tipo celular CD8+ es esencial a la hora de conferir protección frente a múltiples serotipos (Calvo-Pinilla et al., 2012). Aunque las microesferas generadas inducen principalmente una activación de la respuesta T CD4+ (probablemente debido al tamaño de la microesferas, entre 1,5 y 4 µm de diámetro) ya que son presentadas por los MHC de clase II vía endosoma, se pueden diseñar materiales particulados que escapen de las vías endosomáticas y lisosomáticas tras ser internalizadas por las células presentadoras de antígeno (APC) (Beaudette et al., 2009; Shen et al., 2006) y que confieran una potente respuesta mediada por células. Distintos estudios han demostrado de esferas inertes tapizadas con anticuerpos estimulan la respuesta T CD8, siendo el diámetro óptimo para conseguir un mayor estímulo de 1 µm (Falo et al., 1995). Otros materiales particulados como las VLPs, así como muchos virus, tienen un tamaño de entre 0,03 y 0,2 µm de diámetro, o microesferas de poliestireno carboxilado, con un diámetro de entre 0,04 y 0,05 µm. Antígenos conjugados a estas microesferas fueron capaces de inducir tanto una respuesta de tipo humoral como celular T CD8+, comparable a la acción de los adyuvantes que son capaces de activar ambas respuestas inmunes (Fifis et al., 2004). Estos estudios sugieren que la generación de micoresferas basadas en la proteína muNS del ARV con un menor diámetro podría mejorar la activación de la respuesta T CD8+, mejorando a su vez la protección cruzada frente a múltiples serotipos de BTV.

Otra opción para mejorar la protección cruzada de las vacunas basadas en microesferas ha sido combinarlas con vectores virales que mejoren la respuesta inmune celular CD8+ como los MVAs, siguiendo una estrategia de inmunización *prime-boost*. La inmunización primero con microesferas que continen VP2, VP7 y NS1 y una segunda inmunización con rMVAs que expresan estas tres mismas proteínas protegió tanto frente al desafío homólogo con BTV-4 como al heterógo con BTV-1. Esta nueva estrategia combinada indujo niveles similares de anticuerpos neutralizantes específicos para BTV-4 que los alcanzados con la inmunización solo con microesferas. Cabe destacar que el *boost* con los MVAs recombinantes elevó los antcuerpos de tipo Ig2a e IgG2b en suero, aumentando por

tanto la relación IgG2a/IgG1, lo que sugiere que el empleo de rMVA como *boost* estimuló la respuesta inmune celular de tipo Th1. Los distintos isotipos de inmunoglobulinas presentan diferentes funciones en cuanto a inmunidad antiviral. Se ha descrito como las vacunas frente a Influenza estimulan la secreción de las subclases IgG1 e IgG2a, induciendo una mejor protección que aquellas que solo inducían IgG1 con actividad neutralizante (Huber et al., 2006). El aumento de inmunoglobulinas IgG2a está más relacionado con la respuesta celular citotóxica mediada por anticuerpos y respuestas mediadas por complemento (Liu et al., 2016; Yendo et al., 2016), los cuales se han descrito que contribuyen al aclaramiento de la infección en el hospedador (Huber et al., 2001). Aunque no se han realizado estudios sobre el papel de los distintos isotipos en relación con la protección frente a BTV, éstos deberían realizarse para así poder mejorar la eficacia de las vacunas frente a BTV. Se ha demostrado que las respuestas inmunes celulares, especialmente la citotóxica, son importantes en el aclaramiento de las infecciones homólogas y heterólogas producidas por los distintos serotipos de BTV (Jeggo, Wardley, and Brownlie, 1985) y se ha descrito también que los MVAs recombinantes son potentes inductores de la respuesta T CD8 cuando son utilizados como *boost* tras un fuerte *prime* que exprese los mismos antígenos (Cottingham and Carroll, 2013; Whelan et al., 2009). Estudios anteriores de inmunización llevados a cabo en nuestro laboratorio usando MVAs recombinantes como *boost* mostraron que la inmunización heteróloga DNA/rMVA que expresaban las proteínas VP2, VP7 y NS1 de BTV-4 protegían a los ratones inmunizados frente a los desafíos por BTV-4 así como BTV-1 y BTV-8, y redujeron la viremia en ovejas inmunizadas e infectadas con el serotipo heterólogo BTV-8 (Calvo-Pinilla et al., 2014; Calvo-Pinilla et al., 2012). En este trabajo, tras realizar un ensayo de ELISPOT con esplenocitos procedentes de ratones inmunizados con microesferas y rMVAs expresando VP2, VP7 y NS1 se observó una mayor activación de la respuesta inmune celular en comparación con aquellos animales inmunizados solo con microesferas (*prime/boost*). Para analizar más en profundidad el fenotipo de la respuesta inducida por los antígenos de BTV incluídos en esta estrategia vacunal y con el fin de encontrar epitopos T CD8 inmunodominantes, llevamos a cabo un estudio *in silico* e *in vivo* para analizar la presencia de epitopos CD8 en las proteínas VP2 y NS1 de BTV-4. Aunque ya se habían desrito epítopos CD4 y CD8 en la proteína VP7 de BTV-8 tanto en ratón como en oveja (Rojas et al., 2011), nosotros nos centramos en el estudio de VP2 y NS1, en las cuales se han identificado epítopos citotóxicos en ovejas inmunizadas con BTV (Andrew et al., 1995; Janardhana et al., 1999) Ninguno de los seis péptidos identificados como epitopos CD8 mediante el análisis *in silico* de la proteína VP2 fue capaz de estimular la producción de IFNγ en esplenocitos de ratones inmunizados con rMVA-VP2 (*prime/boost*). Por el contrario, uno de los péptidos de NS1, el NS1-152, se identificó como epítopo T CD8, estimulando la expresión de

IFNγ y CD107a (marcador de citotoxicidad) en células T CD8. Este epítopo 152 está conservado entre los diferentes serotipos de BTV y ha sido caracterizado como epítopo celular T en ovejas y ratones C57BL/6 (Rojas et al., 2014). Mediante la técnica de tinción intracelular de citoquinas o ICCS (*Intracellular cytokine staining*) se observó que el péptido NS1-152 inducía la activación de células T CD8 citotóxicas *in vivo* en ratones inmunizados con la estrategia microesfereas/rMVAs, a diferencia de lo observado cuando los animales fueron inmunizados solo con microesferas, donde se activaba principalmente una respuesta T CD4 (Marin-Lopez et al., 2014). Cuando estudiamos la protección cruzada inducida por la estrategia microesferas/rMVAs, todos los animales inmunizados sobrevivieron a la infección con una dosis letal de BTV-1, no observándose viremia.

En resumen, la combinación de microesferas y MVAs recombinantes siguiendo una estrategia *prime-boost* aumenta la respuesta inmune celular CD8 y la capacidad de protección cruzada frente a distintos serotipos de BTV. Esta nueva estrategia experimental es un enfoque atractivo a la hora de generar vacunas efectivas, seguras, marcadoras y multiserotipo frente a BTV.

3. ESTUDIO DE LA CAPACIDAD DE LA PROTEÍNA NO ESTRUCTURAL NS1 DE BTV, CONSERVADA ENTRE LOS 27 SEROTIPOS DESCRITOS, COMO ANTÍGENO INDUCTOR DE RESPUESTA CELULAR Y PROTECCIÓN MULTISEROTIPO FRENTE AL VIRUS.

La protección frente a infecciones virales depende de la acción de múltiples mecanismos inmunes efectores. Por ejemplo, la presencia de una fuerte respuesta de interferón tipo I es esencial para frenar el inicio de la infección como se ha demostrado en cepas de ratones capaces de activar los mecanismos de defensa a través del receptor de interferón (Calvo-Pinilla et al., 2009a). Para el desarrollo de vacunas, activar una respuesta inmune adquirida apropiada es un punto crítico para alcanzar un efecto protector eficaz. En este trabajo hemos generado una vacuna universal frente a BTV, basada exclusivamente en el vector viral MVA recombinante que expresa la proteína no estructural NS1. Esta vacuna induce activación de células T CD8+ citotóxicas, mecanismo principal de protección en el modelo de ratón IFNAR(-/-) frente a múltiples serotipos de BTV (BTV-1, BTV-4, BTV-4M, BTV-8 and BTV-16) sin inducción de anticuerpos neutralizantes. Además, al generar un MVA recombinante que expresa solo la mitad amino-terminal de la proteína NS1, y que denominamos NS1-Nt (rMVA-NS1-Nt) hemos demostrado que esta región de la proteína NS1 (aminoácidos 1 a 270)

es la responsable de tal protección (tabla A alineamiento de secuencias NS1 material suplementario S2 capítulo III).

La función de los anticuerpos neutralizantes en cuanto a protección frente a BTV está profundamente caracterizada. Múltiples estudios han puesto de manifiesto el rol esencial de la proteína de la capa externa del virión VP2 como inductor de anticuerpos neutralizantes específicos de serotipo, usando diferentes plataformas vacunales como vacunas inactivadas y atenuadas, subunidad, vacunas DNA, VLPs, vectores virales o replicones (Calvo-Pinilla et al., 2009b; Huismans et al., 1987; Kochinger et al., 2014; Legisa et al., 2015; Marin-Lopez et al., 2014; Nunes et al., 2014; Parker et al., 1975; Stewart et al., 2013). Dicha protección mediada por anticuerpos neutralizantes también se ha observado en otros orbivirus relacionados con BTV como peste equina africana (*African Horse Sickness Virus*, AHSV) por medio de transferencias pasivas de suero procedentes de animales inmunizados con el vector viral recombinante MVA que expresa la proteína VP2 de AHSV-4 (Calvo-Pinilla et al., 2015) y ocurre en otras tantas infecciones víricas emergentes como Zika, Ébola, fiebre del valle del Rift (RVFV) (Chen et al., 2017; Sapparapu et al., 2016; Warimwe et al., 2016) o fiebre amarilla por dar un ejemplo, donde se transfirió suero de humanos convalecientes expuestos al virus silvestre a monos del género *Rhesus,* quedando protegidos frente a la infección (Stokes, 1997). Esta función protectora mediada por anticuerpos neutralizantes también se ha descrito en el caso de bacterias patógenas, como los anticuerpos neutralizantes dirigidos frente a proteínas de evasión del sistema inmune de *Staphylococcus aureus* (Boerhout et al., 2015; Shannon, Uekotter, and Flock, 2006) o frente al antígeno de superficie OspA de *Borrelia Burgdorferi,* agente causal de la enfermedad de Lyme (Fikrig et al., 1990).

Aunque las vacunas que inducen anticuerpos neutralizantes frente a la proteína VP2 de BTV protegen frente a la infección por este virus, éstas son específicas de serotipo. La necesidad de encontrar vacunas capaces de inducir respuestas inmunes mediadas por células T CD8+ citotóxicas frente a las proteínas conservadas entre serotipos de BTV y por tanto, de encontrar una vacuna universal frente a cualquier serotipo de este virus, va en concordancia con estudios que muestran la importancia crítica de este tipo de respuesta inmune celular a la hora de conseguir protección multuserotipo frente a BTV (Anderson et al., 2014a; Anderson et al., 2014b; Jones et al., 1996).

En este trabajo demostramos cómo con un solo antígeno de BTV, la proteína NS1 altamente conservada entre serotipos, expresada en el vector viral MVA, es capaz de conferir protección frente a la infección por distintos serotipos de BTV siguiendo una estrategia de

inmunización *prime-boost,* induciendo una potente respuesta T citotóxica. Trabajos previos han descrito esta proteína como el principal inmunógeno inductor de la activación de CTLs (Anderson et al., 2014a; Anderson et al., 2014b). Por esta razón, hemos estudiado las regiones de NS1 responsables de la inducción de la respuesta inmune celular. La región de la proteína NS1 que contiene la mayor parte de los epítopos B, se localiza próxima al extremo carboxilo-terminal (Monastyrskaya, Gould, and Roy, 1995), mientras que la región que abarca la mayor parte de los epítopos T CD8 teóricos se encuentra en la mitad amino-terminal.

En este trabajo se han generado dos rMVAs, uno que expresa la primera mitad de la proteína NS1 (NS1-Nt) y otra que expresa la segunda mitad (NS1-Ct) con el fin de analizar su uso potencial como vacunas frente a BTV. Nuestros resultados mostraron que el rMVA-NS1-Ct estimulaba una producción alta de anticuerpos pero una escasa respuesta inmune celular y esta construcción no fue capaz de proteger a los animales inmunizados frente a la infección con una dosis letal de BTV-4. Sin embargo, sí observamos un retraso en la mortalidad de los animales inmunizados con rMVA-NS1-Ct comparándolos con los no inmunizados. Este retraso posiblemente sea debido a los anticuerpos inducidos por esta vacuna que, aun no teniendo actividad neutralizante, sí podrían estar involucrados en otros procesos como opsonización, pero se requerirán estudios más profundos que lo confirmen. Sorprendentemente, los animales inmunizados con rMVA-NS1-Nt sobrevivieron a la infección frente a BTV-4 e incluso frente al resto de serotipos de BTV utilizados en este trabajo. Los títulos de anticuerpos observados en estos animales fueron bajos y sin capacidad neutralizante, pero sí que se observó un fuerte aumento de la respuesta T CD8+ citotóxica. Estos resultados apoyan que la proteína NS1, en concreto, su región amino-terminal, es necesaria y suficiente para proteger a los animales, sugiriendo que las células CD8+ citotóxicas desempeñan un papel esencial para conseguir dicha protección, y siendo un prometedor candidato vacunal universal frente a múltiples serotipos del virus de la lengua azul.

Además hemos demostrado que el efecto protector de la proteína NS1 reside en un epítopo T CD8 inmunodominante de su secuencia, el péptido 152. Con el fin de demostrar esta afirmación, llevamos a cabo la deleción del péptido 152 en la secuencia de NS1-Nt, generando así el MVA recombinante rMVA-NS1-NtΔ152, el cual pierde su capacidad protectora cuando los ratones son inmunizados y desafiados con BTV. También se observó un retraso en la mortalidad de estos animales en comparación con los controles no inmunizados, lo que podría sugerir que otros péptidos no inmunodominantes podrían desempeñar alguna función en esta protección parcial. Los datos obtenidos en este estudio sirven para fomentar el desarrollo de nuevas estrategias vacunales que induzcan potentes respuestas T frente a BTV basadas en este

péptido, el 152 (el cual está altamente conservado en la mayoría de serotipos de BTV) con el fin de alcanzar una eficacia protectora máxima.

Por último nos centramos en la investigación de los componentes del sistema inmune relacionados con la protección generada por rMVA-NS1. Analizamos si las células CD8+ o el suero estaban involucrados en la protección frente al virus a través de ensayos de transferencia adoptiva. Se transfirieron fracciones enriquecidas de células CD8+ o suero procedente de animales inmunizados con MVA-NS1 a animales *naive* que posteriormente fueron desafiados con una dosis letal de BTV-4. Observamos una protección parcial dependiente de dosis en los animales transferidos con células CD8+ procedentes de animales inmunizados con rMVA-NS1, mientras que no observamos ningún efecto en los animales transferidos con suero en cuanto a protección. Los datos obtenidos del ensayo de transferencia de suero se apoyan en los resultados de otros trabajos descritos en la literatura, donde se observa que la protección debida a la transferencia de suero desde animales inmunizados queda completamente abolida cuando se emplea una dosis letal del virus (Goncalves et al., 2015), sugiriendo que los anticuerpos específicos de NS1 no son capaces por si solos de controlar la infección cuando la carga viral es alta. Por otra parte, hemos observado que las células CD8+ proporcionan una protección parcial dependiente de dosis frente al desafío letal. Experimentos similares demuestran la función esencial de las células CD8+ y la dependencia del número de células transferidas (Champagne et al., 2016; Elong Ngono et al., 2017; Martins et al., 2016; Xu et al., 2017). Con este experimento de transferencia de células CD8+, demostramos la participación de esta población celular en la protección frente a BTV, que por medio de su actividad citotóxica contribuyen al control de la carga viral.

La lengua azul es una importante enfermedad reemergente en el área de la salud animal y que afecta a todos los continentes habitados. Con el fin de controlar la expansión del virus que lo provoca, BTV, se requiere el desarrollo de vacunas multiserotipo eficientes que permitan conseguir protección frente a todos los serotipos y la diferenciación entre animales infectados y vacunados. Nuestros resultados iniciales aquí descritos ponen de manifiesto la importancia de la proteína NS1 en cuanto a su función protectora mediada por células T CD8+ frente a múltiples serotipos de BTV, cuando es vehiculizada en el vector viral recombinante MVA, y también que esta función protectora se encuentra en la región amino-terminal. A esto hay que añadir que un sistema de ELISA basado en el reconocimiento de la proteína NS1, en combinación con los ensayos diagnósticos comerciales basados en la proteína VP7, sería una gran herramienta para diferenciar entre los animales infectados de manera natural y los

vacunados con rMVA-NS1, debido al alto nivel de anticuerpos específicos de NS1 que induce esta vacuna.

Concluimos por lo tanto que rMVA-NS1 así como rMVA-NS1-Nt son unos prometedores candidatos vacunales universales frente a BTV. Además confirmamos la importancia del epítopo inmunodominante 152 para conferir capacidad protectora a NS1 y NS1-Nt y el papel importante que las células T CD8+ desempeñan en la protección que confiere la vacuna rMVA-NS1 frente al virus de la lengua azul.

CONCLUSIONES

1. Se ha caracterizado la patología producida por BTV, en concreto por la cepa BTV-4 (MOR2009/09), en el modelo murino adulto de laboratorio IFNAR(-/-). Además de ser susceptible a la infección, este modelo reproduce muchas características de la enfermedad observada en el hospedador natural, como son los signos clínicos, el tropismo celular y tisular, las lesiones patológicas, cambios en los parámetros hematológicos, producción de citoquinas proinflamatorias durante el curso de la infección en tejidos linfoides, daño celular debido tanto a la replicación del virus como a los procesos de apoptosis activados por la respuesta proinflamatoria y la relación de estos cambios con la patología descrita. Por último, se ha demostrado la gran utilidad de este modelo para el estudio de la trasmisión de BTV, su patogénesis y virulencia así como su adecuación para el ensayo de vacunas.

2. Se han generado vacunas marcadoras recombinantes frente a BTV basadas en microesferas compuestas por la fracción mínima de la proteína muNS de ARV (muNS-Mi) que incorporan los antígenos de BTV-4 VP2, VP7 y NS1. La vacunación de ratones IFNAR(-/-) con esta plataforma siguiendo una estrategia de inmunización *prime-boost* confiere protección total frente al desafío homólogo con BTV-4 y parcial frente al desafío heterólogo con BTV-1, induciendo anticuerpos neutralizantes y una respuesta inmune celular T principalmente de tipo CD4.

3. La vacunación combinada con microesferas y MVAs recombinantes, que expresan los antígenos VP2, VP7 y NS1, confieren protección total en ratones IFNAR(-/-) frente al desafío tanto homólogo con BTV-4 como heterólogo con BTV-1, alcanzando niveles de anticuerpos neutralizantes semejantes a los alcanzados con la vacunación *prime-boost* homóloga de microesferas y mejorando la respuesta inmune celular T CD8, implicada en la protección multiserotipo frente a BTV.

4. Se ha descrito un epítopo T CD8 en la proteína NS1 de BTV, epítopo altamente conservado en los 27 serotipos del virus. Se ha confirmado que el péptido 152 (GQIVNPTFI) de la proteína NS1 es capaz de activar una respuesta CD8 citotóxica en esplenocitos de animales inmunizados con MVA-NS1.

5. La proteína NS1 de BTV-4 vectorizada en MVA recombinantes induce una fuerte respuesta inmune celular citotóxica CD8 y confiere entre el 80 y el 100% de protección frente a los desafíos con dosis letales de los serotipos 1, 4, 4M, 8 y 16 de BTV en ausencia de anticuerpos con actividad neutralizante. Además, esta capacidad antigénica protectora de la proteína NS1 de BTV reside en su región amino-terminal (aminoácidos 1-270).

6. La respuesta T CD8 protectora inducida por la proteína NS1 requiere de la presencia del epítopo T denominado 152, epítopo inmunodominante cuya deleción suprime la capacidad protectora de esta proteína.

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ANEXO

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Interferon α/β receptor knockout mice as a model to study bluetongue virus infection

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ABSTRACT

Bluetongue is an arthropod-borne disease caused by a virus of the genus *Orbivirus*, the bluetongue virus (BTV), which affects ruminant livestock such as cattle, sheep, and goats and wild ruminants such as deer, and camelids. Recently, adult mice with gene knockouts of the interferon α/β receptor (IFNAR−/−) have been described as a model of lethal BTV infection. IFNAR(−/−) mice are highly susceptible to BTV-1, BTV-4 and BTV-8 infection when the virus is administered intravenously or subcutaneosuly. Disease progression and pathogenesis closely mimics signs of bluetongue disease in ruminants. In the present paper we review the studies where IFNAR(−/−) mice have been used as an animal model to study BTV transmission, pathogenesis, virulence, and protective efficacy of inactivated and new recombinant marker BTV vaccines. Furthermore, we report new data on protective efficacy of different strategies of BTV vaccination and also on induction of interferon α/β and proinflammatory immune responses in IFNAR(−/−) mice infected with BTV.

1. IFNAR(−/−) mice as a model to study viral infections

Blocking IFN-α/β activity in mice leads to a dramatically increased sensitivity to many viruses. Mice lacking the type I interferon (IFN) receptor (IFNAR (−/−)) were generated to elucidate the physiological role of the type I IFN system by Müller and colleagues (Muller et al., 1994). These mice were unresponsive to the antiviral action of natural murine type I IFN, a mixture of IFN-α and IFN-β. Comparative cytofluorometry revealed no abnormalities in the major lymphocyte subsets in terms of expression of CD3, CD4, CD8 (thymocytes and splenocytes) and major histocompatibility complex (MHC) class I and class II antigens (thymocytes, splenocytes, and peritoneal macrophages). IFNAR(−/−) mice showed no overt anomalies but were unable to cope with viral infections.

The lack of a type I IFN system allows the virus to replicate more efficiently and IFNAR(−/−) mice have been used as a laboratory animal model to study the immune response, determinants of virulence, vaccine development, or pathogenicity of Crimean-Congo hemorrhagic fever virus (Bereczky et al., 2010; Zivcec et al., 2013), human T cell leukemia virus type I (Delebecque et al., 2005), Schmallenberg virus (Wernike et al., 2012), West Nile virus (Winkelmann et al., 2012), poliovirus (Ida-Hosonuma et al., 2005; Ohka et al., 2007), Theiler's virus (Fiette et al., 1995), La Crosse virus (Pavlovic et al., 2000), measles virus (Mrkic et al., 1998; Volker et al., 2013), hepatitis B and C viruses (Aly et al., 2011; Chen et al., 2013), or Rift valley fever virus (Bouloy et al., 2001; Lopez-Gil et al., 2013; Lorenzo et al., 2010). All these data, and the presence of an otherwise intact immune system in these mice suggest that IFNAR(−/−) mice could be a good animal model to study bluetongue virus (BTV) infections and to evaluate vaccine strategies against this virus.

2. Bluetongue and type I interferon

The innate immune response is the first line of defense against viruses resulting in the production of IFNα/β and other pro-inflammatory cytokines that control de infection (Randall and Goodbourn, 2008). BTV infection induces type I IFN in cells in culture and ruminants (Foster et al., 1991; Fulton and Pearson, 1982; Huismans, 1969; Jameson and Grossberg, 1978, 1981; MacLachlan and Thompson, 1985). Although the induction of type I IFN after BTV infection was described more than 40 years ago, the mechanism of IFNα/β induction has remained unknown for several years. Recent studies showed that BTV induced IFN-α/β in skin lymph and in blood in vivo (Ruscanu et al., 2012). Although BTV replicated in a substantial fraction of the conventional dendritic cells (cDCs) and plasmacytoid dendritic cells (pDCs) in vitro, only pDCs responded to BTV by producing a significant amount of IFN-α/β. BTV replication in pDCs was not mandatory for IFN-α/β production since it was still induced by UV-inactivated BTV, and the induction of IFN-α/β in

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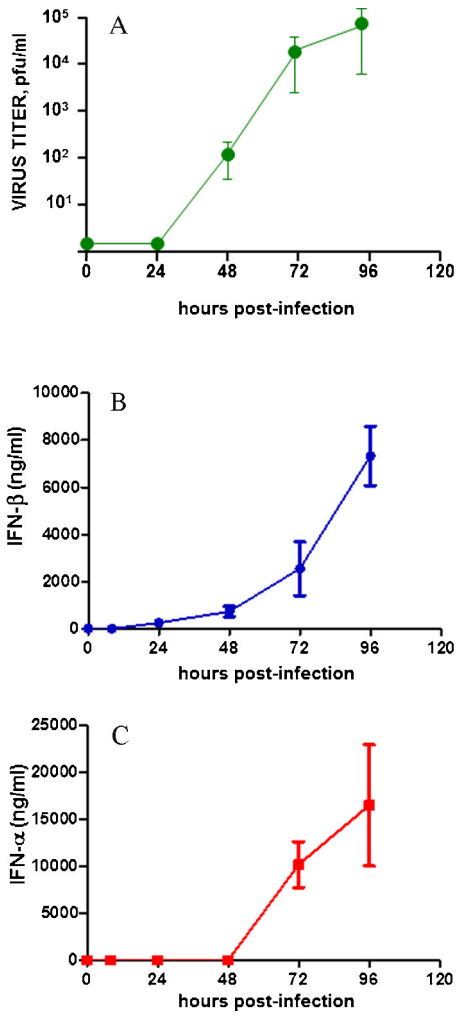

Fig. 1. Production of IFN-α and IFN-β measured in serum of IFNAR(−/−) mice infected with BTV-4. Mice were infected subcutaneously with 10^3 PFU per mouse of BTV-4 (MOR2009/09) (A) Titers of virus recovered in blood after infection. Virus was extracted from blood and the titer was determined by plaque assay in Vero cells. Each point represents the mean values of the viral titer of five animals, and standard deviations are shown as error bars. (B) Kinetic of IFN-β production in serum of infected mice detected by ELISA (Verikine Mouse IFN-β ELISA kit, PBL interferon source, USA). (C) Kinetic of IFN-α production in serum of infected mice detected by ELISA (Verikine Mouse IFN-α ELISA kit, PBL interferon source). Levels of IFN-α and IFN-β were measured for each group of five mice in pools of sera collected at 12, 24, 48, 72, or 96 h.p.i., by ELISA.

pDCs occurred via a novel TLR-independent and Myd88-dependent pathway (Ruscanu et al., 2012). However, this is not the only way of IFN-α/β induction. Another study showed that both viral RNA sensors RIG-I and MDA5 are upregulated by BTV infection of the epithelial bronchial human cell line A549 and that viral recognition by RIG-I and MDA5 is the driving force for the activation of IFN regulatory factor 3 (IRF3) and consequently the induction of IFN-α/β. In addition, this study showed that virus replication is essential for IFN-β induction in A549 cells (Chauveau et al., 2012).

These two different ways of type I IFN induction can explain the fact that BTV is a potent interferon inducer in wild type mice without virus replication (Jameson et al., 1978) but in IFNAR(−/−) mice there is a strong correlation between BTV replication, viremia, and the induction of IFN-α and IFN-β. The kinetics of IFN-α and IFN-β production measured in sera of IFNAR(−/−) mice infected subcutaneously with 10^3 PFU of BTV-4 (MOR2009/09) per mouse are shown in Fig. 1. IFN-β is detected in serum at 48 h post-infection when BTV is detected at low levels in blood (titers up to 10^2 PFU/ml). IFN-α is detected in serum at 72 h post-infection when the viremia is in the upper level (titers up to 8×10^4 PFU/ml). In both cases, the production of IFN increases to a maximum at 96 h post-infection, before the death of the animals. The IFN production peak also coincides with the viremia peak in sheep infected with several strains of BTV and this high concentration of IFN induces a decrease in the viremia of the infected animals (Foster et al., 1991). Although the infection of IFNAR(−/−) mice with BTV induces a strong production of IFN-α/β in response to viral infection, the absence of receptor in these animals does not allow the type I IFN signal transduction and the antiviral defense, increasing the susceptibility of IFNAR(−/−) mice to viral infections.

3. IFNAR (−/−) mice as a model to study BTV virulence

Bluetongue (BT) is an arthropod-borne disease caused by a virus of the genus *Orbivirus*, the BTV, which affects ruminant livestock such as cattle, sheep, and goats and wild ruminants such as deer, and camelids. For years, different groups have tried to establish a laboratory animal model to facilitate the studies of pathogenesis, immune response and vaccination against BTV. Natural hosts are expensive and require specialized animal facilities with biosafety level 3 for these studies. It is known that BTV infects mouse embryos (Bowen et al., 1982). Furthermore, experimental studies showed that BTV grew in suckling mice inoculated intracerebrally and the growth was faster in mice at 1 day than at 2 weeks of age (Narayan and Johnson, 1972). In contrast, adult mice are nor susceptible to BTV infection and viremia is not observed in mice inoculated either intravenously or subcutaneously (Calvo-Pinilla et al., 2009a). Previous studies showed that lesions caused by bluetongue virus infection of the central nervous system in sheep and mice vary with age of the host suggesting that the character of the lesions appears to be influenced by the stage of immunological maturity of the infected animals (Richards and Cordy, 1967). All these studies suggest that the cellular receptor is not a limitation for BTV to infect mice and the possible limitation for the productive infection in mice could be the innate immune response against BTV generated for the animal that establishes an antiviral state.

Recently, our laboratory has shown that adult mice deficient for type I IFN receptor (IFNAR(−/−)) are highly susceptible to infection with serotypes 4 (BTV-4) and 8 (BTV-8) of BTV when the virus is administered intravenously (Calvo-Pinilla et al., 2009a). The fisrt characterization of this model was developed in IFNAR(−/−) mice with a C57BL/6 genetic background and intravenously infected. Afterwards, we observed that IFNAR(−/−) mice with a 129 genetic background showed the same susceptibility to BTV infection and no differences were found between subcutaneous and intravenous infection in the survival rates and appearance of clinical signs and viremia (Calvo-Pinilla et al., 2009b, 2012). Recently, we have also characterized this murine model for serotype 1 (BTV-1). IFNAR(−/−) mice infected with serotypes 1, 4 or 8 showed the same clinical signs characterized by ocular discharges, apathy and the disease progression led to animal death. Infectious virus was recovered from the spleen, lung, thymus, lymph nodes and blood. The appearance of viremia after BTV infection in IFNAR(−/−) mice was dependent on the viral dose, althought the highest titers observed in blood were not dose-dependent (Fig. 2). The differential virulence of serotypes 1, 4, and 8 was maintained in this animal model. Some BTV serotypes such as serotype 8 exhibit enhanced virulence in cattle (Saegerman et al., 2008), in contrast to BTV-4 that usually exhibits subclinical infections in this species. In infected IFNAR(−/−) mice, BTV-1 and BTV-8 killed 100% of the animals with a dose as low as 10 PFUs per mouse. In contrast 10^3 PFUs of BTV-4 were needed to kill 100% of the mice. The highest titers of virus

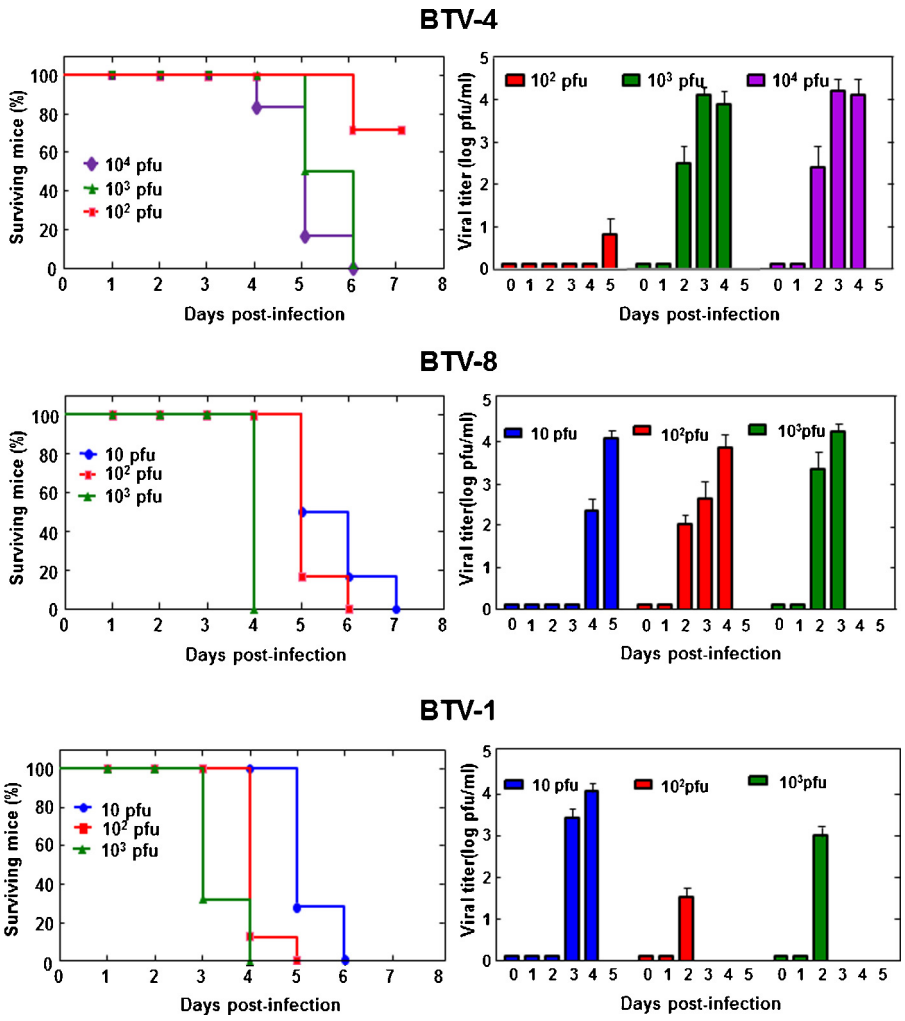

Fig. 2. Susceptibility of adult mice to BTV-1, BTV-4, and BTV-8 infection. IFNAR(−/−) mice (8 weeks old, 5 mice per group) were subcutaneously inoculated with different PFUs of BTV-4 (A), BTV-8 (B), and BTV-1 (C). Survival rates of IFNAR(−/−) mice and viremia are shown. The number of PFUs inoculated is indicated on each survival group. The mice were observed every 24 h for 7 days.

found in the blood of infected mice were similar for these three serotypes of BTV (Fig. 2), indicating that BTV-1 exhibits higher virulence than BTV-4 in the IFNAR(−/−) model as it was previously described for BTV-8 (Calvo-Pinilla et al., 2009a). Experimental studies performed in BTV natural hosts have proved a greater virulence of BTV-1 in sheep. BTV-1 infected sheep show a longer clinical course, with a significant increase in clinical signs, as well as more severe gross lesions than BTV-8 infected sheep. These differences appear not to be attributable to a greater virus replication, since viremia was lower in BTV-1 infected sheep, suggesting that viral loads did not influence the pathogenicity of these serotypes (Sanchez-Cordon et al., 2013b). The higher virulence of BTV-1 in sheep, as compare to BTV-8, despite lower viremia values, is confirmed by experimental results in IFNAR(−/−) mice (Fig. 2). These observations indicate that IFNAR(−/−) mice could be an adequate small animal model to study differences in virulence among BTV serotypes because BTV infection in these mice reproduces viral virulence observed in the natural host. In this direction, IFNAR(−/−) mice have been used recently to analyze the determinants of virulence of BTV field strains maintained at either low (L) or high (H) passage number in cell culture (Caporale et al., 2011). The L strains were lethal for IFNAR(−/−) mice while the virulence of the H strains were significantly attenuated in this experimental model, as it also observed in the BTV natural host.

4. IFNAR(−/−) mice as a model to study BTV transmission

BTV is transmitted among ruminants predominantly through feeding of biting midges that are members of the genus *Culicoides* (Verwoerd, 2004). Besides ruminants, natural BTV infection among African carnivores as a result of oral infection (Alexander et al., 1994), as well as the deaths of two Eurasian lynx caused by BTV-8 after feeding ruminant fetuses (Jauniaux et al., 2008) have been reported. These data suggested the possibility of oral transmission of BTV. IFNAR(−/−) mice were used to confirm this route of infection in a systematic way. In this study (Calvo-Pinilla et al., 2010), the capacity of BTV-8 to infect IFNAR(−/−) mice by the oral route was demonstrated. The esophagus and the oral cavity were susceptible to BTV infection and replication. Furthermore, in IFNAR(−/−) mice, oral infection produced similar levels of viremia, clinical signs and tissue lesions to those observed after intravenous infection (Calvo-Pinilla et al., 2010). All these data showed that, in principle, BTV could infect animals using the oral route and that IFNAR(−/−) mice can be a good model to study routes of BTV transmission.

5. IFNAR(−/−) mice as a model to study BTV pathogenesis

IFNAR(−/−) mice can be also useful for investigating various facets of BTV-host interaction, including virus pathogenesis. The

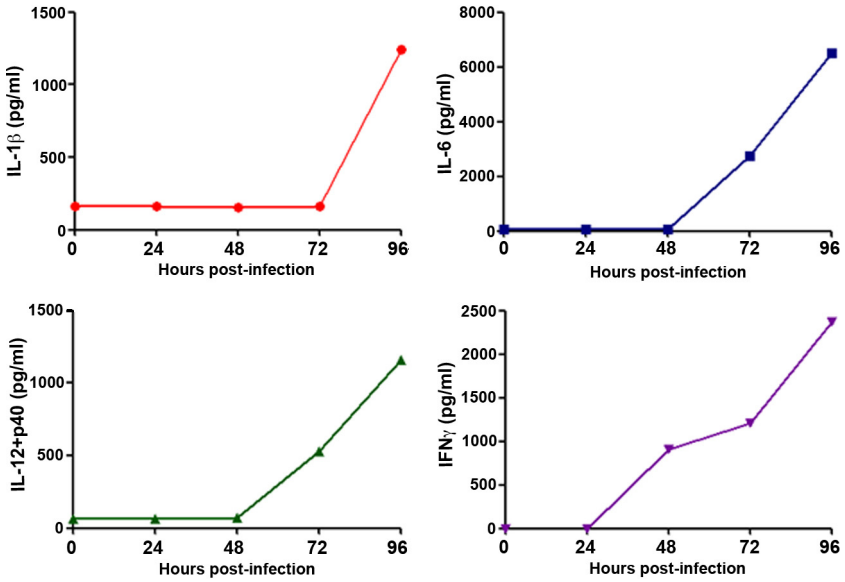

Fig. 3. Production of pro-inflammatory cytokines measured in serum of IFNAR(−/−) mice infected with BTV-4. Mice were infected subcutaneously with 10^3 PFU per mouse of BTV-4 (MOR2009/09). Serum levels of IL-1β, IL-6, IL-12 p40, and IFN-γ were measured for each group of five mice in pools of sera collected every 24 h after infection by using specific ELISA (Invitrogen).

innate immune response elicited by dsRNA viruses, including the production of type I IFN (alpha/beta) and other inflammatory cytokines are likely to be key factors in the expression of their variable pathogenicity levels (Johansson et al., 2007). Experimental studies have been conducted to examine the expression of pro-inflammatory cytokines (IL-1, IL-6, IL-12, IFN-γ and TNF-α) and pathology of bluetongue in sheep and goats infected with BTV. The experimental findings have highlighted the involvement of pro-inflammatory cytokines (IL-12, IFN-γ and TNF-α), vasoactive substances produced in macrophages, and CTLs in BTV pathogenesis and viral kinetics. Thus, the endothelial injury observed during BTV infection could be due to the direct pathogenic effect of BTV infection on endothelial cells or to a response to inflammatory mediators released by virus-infected endothelial cells and, possibly, other cell types such as monocytes/macrophages (Channappanavar et al., 2012; Drew et al., 2010; Hemati et al., 2009; Sanchez-Cordon et al., 2013a). Due to de absence of tools that allow the detection of most of the ruminant cytokines in serum, these experimental studies estimate the cytokine mRNA by quantitative real time PCR (Channappanavar et al., 2012; DeMaula et al., 2002; Ruscanu et al., 2012; Sanchez-Cordon et al., 2013a; Umeshappa et al., 2010, 2011). A tremendous advantage of the IFNAR(−/−) mouse model is the availability of a wide variety of reagents that can be used to study many aspects of the immune response to the virus. We have analyzed whether the up-regulation of pro-inflammatory cytokines described in ruminants infected with BTV corresponds with an induction of these cytokines in the IFNAR(−/−) mice infected with BTV-4 (MOR2009/09), even though their innate immune response is compromised. IFNAR(−/−) mice developed strong proinflammatory immune responses following BTV-4 infection, as demonstrated by increases in IL-6, IL-12, and IL-1β concentrations. IL-6, IL-12, and IFN-γ were detected in the sera 48 h post-infection and IL-1β at 72 h post-infection (Fig. 3). The appearance and accumulation of these cytokines in BTV infected IFNAR(−/−) mice correlates with the presence of the virus in blood (Figs. 1 and 3), suggesting continuous overstimulation of the innate immune system by BTV-4 replication. Thus, the maximum concentration of cytokines in serum correlates with evident gross pathological alterations characterized by widespread edema, hemorrhages especially in spleen and lungs, and enlarged spleen and lymph nodes (Calvo-Pinilla et al., 2009a, 2010). Similar alterations have been described in BTV infected ruminants, including permeability disorders in the vascular system (Barratt-Boyes and MacLachlan, 1994; MacLachlan et al., 1990, 2009; Schwartz-Cornil et al., 2008).

The histological examination of IFNAR (−/−) mice infected with BTV-4 showed also similar pathological alterations compared to BTV infected ruminants (MacLachlan et al., 2008, 2009; Worwa et al., 2010). Lungs of BTV infected mice showed hyperemia and increased septum size with infiltration of lymphocytes, macrophages and a few neutrophils, histopathological findings consistent with bronchointerstitial pneumonia. It was also observed the presence of a moderate edema in the alveolar cavity with presence of abundant alveolar macrophages and a few detached epithelial cells (descamative alveolitis) (Fig. 4). The infected spleen showed a marked lymphoid depletion with infiltration of neutrophils in the white pulp. This lymphoid depletion was also observed in lymph nodes and in the thymus as well as the loss of thymic architecture with the medulla and the cortex becoming not distinguishable (Calvo-Pinilla et al., 2009a, 2010) (Fig. 4). These results suggest that BTV-4 produces similar tissue lesions in IFNAR(−/−) mice to those in the natural host and this animal model could be a useful tool to study the pathogenesis produced by BTV.

6. IFNAR(−/−) mice as a model of infection for BTV related orbivirus

After the characterization of BTV infection in IFNAR(−/−) mice, these animals have also been used as a model of infection for African horse sickness virus (AHSV) and epizootic hemorrhagic disease virus (EHDV), two BTV related orbivirus. AHSV-4 infects IFNAR(−/−) mice and the pathology, with the exception of the central nervous system lesions, resemble those found in AHSV infected horses. Hemorrhages and inflammatory changes in the lung, splenomegalia and congestion of other internal organs such as the liver are common pathological findings in horses infected with AHSV (Castillo-Olivares et al., 2011; Mellor and Hamblin, 2004). Furthermore, AHSV infected IFNAR(−/−) mouse tissues showed expression of AHSV antigens, particularly spleen, suggesting that this organ contains a high viral load. This feature is probably

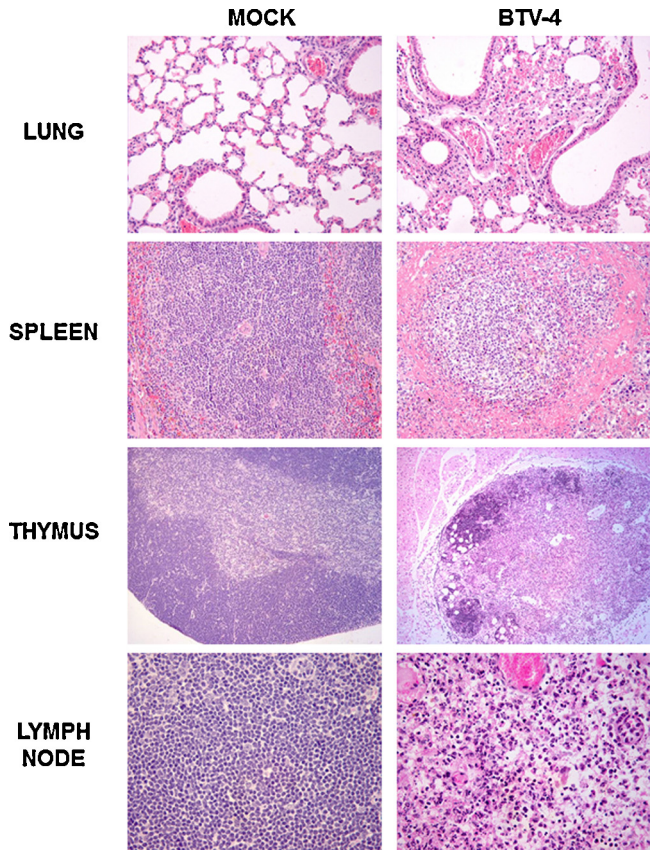

Fig. 4. Tissue sections of BTV-4 infected adult IFNAR(−/−) mice. Mice were infected with 10^3 PFUs of BTV-4 subcutaneously. Tissues were harvested at 48 h.p.i. Hematoxylin and eosin staining are shown.

shared with AHSV infected horses, since the spleen is the preferred organ for making an etiological diagnosis of AHS in the horse postmortem. Recently, the IFNAR(−/−) mouse model of infection has been characterized for a different serotype of AHSV, serotype 9. Animals infected with AHSV-9, showed milder clinical signs than AHSV-4 and the mortality rate was very low. In contrast, the level of viremia was similar in the animals infected with the serotypes 4 or 9, although the period of viremia was shorter in mice infected with AHSV-9 (de la Poza et al., 2013).

IFNAR(−/−) have also been proposed as an animal laboratory model to study EHDV infection. These mice are susceptible to EHDV-7 and the course of the disease is more similar to AHSV than BTV in IFNAR(−/−) mice (Eschbaumer et al., 2012). For serotype 7, the outcome of EHDV infection of IFNAR(−/−) mice is dose-dependent and subclinical infections can occur. The mortality rates, clinical signs and viremia levels of both AHSV serotypes and EHDV-7 are appropriate to use IFNAR (−/−) mice for studies of pathogenesis and vaccine efficacy.

7. IFNAR(−/−) mice as a model to study vaccine efficacy

The cost of testing new vaccines in target species is a major obstacle for laboratories and industries. For this reason, the intracerebral inoculation of newborn mice with BTV vaccines has been used as an animal model to evaluate the level of attenuation of live attenuated BTV vaccines (Franchi et al., 2008). Several studies have shown that adult IFNAR(−/−) mice serve as a good animal model to test BTV vaccines. Even though the lack of type I interferon signals may have an effect in the development of acquired immune responses, the IFNAR(−/−) infection model is useful for the definition of effective vaccine candidates against BTV. This murine model has been previously used to study the efficacy of vaccines against pseudorabies virus (Tudor et al., 2001), Rift Valley virus (Boshra et al., 2011; Lorenzo et al., 2010), rotavirus (Vancott et al., 2003), or La Crosse virus and influenza A virus (Operschall et al., 1999).

To provide further proof that IFNAR(−/−) mice are a good animal model for BTV vaccination studies, vaccination protection experiments were performed with ZULVAC-4 (1.5×10^6 $TCID_{50}$BTV-4) inactivated BTV-4 preparation (Fort Dodge Veterinaria, S.A.). In this study all immunized animals developed an antibody response specific of BTV-4 with the production of neutralizing antibodies against serotype 4, indicating a successful immunization. The protection mediated by inactivated whole BTV vaccine in IFNAR(−/−) mice infected with a lethal dose of BTV-4 was complete, and 100% of the animals did not show any symptoms associated with infection or died (Calvo-Pinilla et al., 2009a). Recently, we have developed in the laboratory the same study of vaccination with an inactivated vaccine and protection with a more virulent serotype, BTV-8. For this purpose, IFNAR(−/−) mice were vaccinated with ZULVAC-8 ($10^{7.5}$ $TCID_{50}$BTV-8) inactivated BTV-8 preparation. All immunized animals developed neutralizing antibodies against serotype 8, and the protection after a lethal dose of BTV-8 was complete. After challenge with BTV-8, clinical signs or death were not observed in the immunized mice, confirming the efficacy of the vaccine and the utility of the mouse model in the studies of vaccine potency test and efficacy.

IFNAR(−/−) mice have been used to facilitate the studies of protection and immune response conferred by recombinant marker vaccines against BTV. The efficacy as a vaccine against BTV of the viral vectors bovine herpesvirus 4 (BoHV-4) and equine herpesvirus type 1 (EHV-1) expressing BTV-8 proteins has been analyzed in IFNAR(−/−) mice (Franceschi et al., 2011; Ma et al., 2012). Mice intraperitoneally inoculated twice with BoHV-4-VP2 developed neutralizing antibodies against BTV-8 and showed a strongly reduced viremia and a longer survival time when challenged with a lethal dose of BTV-8 (Franceschi et al., 2011). Similar results in protection and induction of neutralizing antibodies against BTV-8 were observed in IFNAR(−/−) mice immunized with EHV-1 expressing VP2 and VP5 (Ma et al., 2012).

In our laboratory, several vaccine vectors and strategies of vaccination have been analyzed. We have developed marker recombinant vaccines based on naked DNA and modified vaccinia virus Ankara (rMVA) expressing BTV-4 proteins. To analyze the best vaccination strategy, mice were immunized with an homologous prime-boost of DNA or rMVA or with an heterologous prime-boost of DNA and rMVA. For these studies, the DNA and rMVA expressed VP2, VP5, and VP7 proteins of BTV-4. Although IFNAR(−/−) mice immunized with DNA/DNA or MVA/MVA were partially protected against a challenge with a lethal dose of BTV-4, only the strategy based on heterologous prime-boost of DNA and rMVA completely protected the mice against the BTV-4 challenge (Fig. 5). A delay in the appearance of virus in blood was observed in mice immunized with the strategy based on homologous prime-boost with DNA or MVA but viremia was not detected in the mice immunized with the heterologous prime-boost DNA/MVA strategy that were completely protected (Fig. 5). Neutralizing antibodies play an important role in protective immunity to BTV (Jeggo et al., 1984b). In order to analyze which strategy is inducing stronger neutralization activity against BTV-4, the presence of neutralizing antibodies in sera has been also analyzed in IFNAR(−/−) mice immunized with these three strategies. Homologous prime-boost of DNA expressing VP2, VP5, and VP7 proteins of BTV-4 did not induce neutralizing antibodies; however, the homologous prime-boost of MVA and the heterologous prime-boost DNA/MVA strategies induced high levels of neutralizing antibodies (Fig. 5). The stronger neutralization activity against BTV-4 of the three analyzed strategies corresponded with the more efficient strategy in protection against BTV-4, the

Group	1st Immunization day 0	2nd Immunization day 14	Challenge day 28
1	pcDNA3-VP2/VP5/VP7	pcDNA3-VP2/VP5/VP7	BTV-4
2	rMVA-VP2/VP5/VP7	rMVA-VP2/VP5/VP7	
3	pcDNA3-VP2/VP5/VP7	rMVA-VP2/VP5/VP7	
4	pcDNA3	MVA	

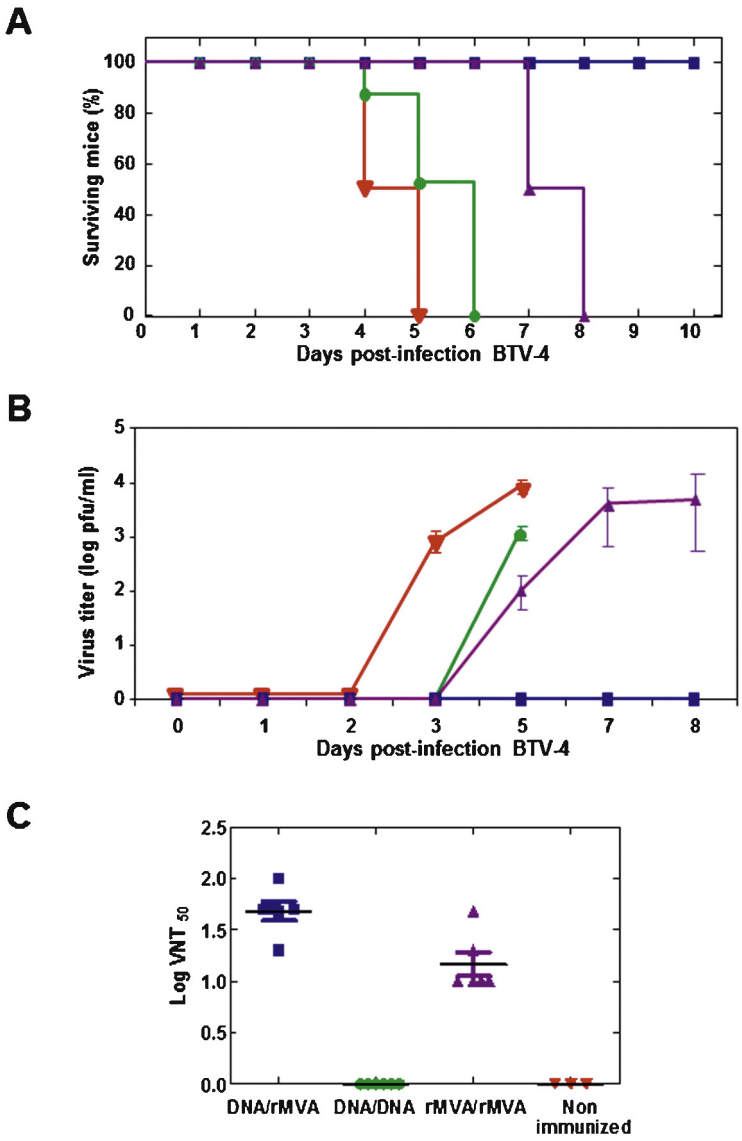

Fig. 5. Analysis of vaccination strategies in IFNAR(−/−) mice immunized with an homologous prime-boost of DNA or rMVA or with an heterologous prime-boost of DNA and rMVA. Mice (8 weeks old, 6 per group) were immunized twice by prime boost vaccination with DNAs (●), rMVAs (▲) or DNA/rMVA (■) expressing VP2, VP5, and VP7 BTV-4 proteins or non-immunized (▼). Two weeks after immunization mice were subcutaneously inoculated with 10^3 PFUs (lethal dose) of BTV-4. (A) Survival rates of immunized and non-immunized IFNAR(−/−) mice after inoculation with BTV-4. The mice were observed every 24 h for 12 days. (B) Titers of BTV-4 recovered in blood of immunized and non-immunized IFNAR(−/−) mice after challenge. Each point represents the mean values of the viral titer of six animals, and standard deviations are shown as error bars. (C) BTV-4 neutralizing antibody detection in VP2/VP5/VP7 immunized mice by VNT at day 28 post-vaccination. Means are presented (—) and standard deviations are shown as error bars.

heterologous prime-boost DNA/MVA. For this reason this strategy has been chosen to identify the best BTV antigens that induce protection against multiple serotypes of BTV.

In addition to neutralizing antibodies, cytotoxic T lymphocytes (CTL) are important in the induction of protective immunity against BTV (Jeggo et al., 1984a). For this reason, the BTV antigens included in a vaccine composition should stimulate humoral and cellular immune response. IFNAR(−/−) mice have been used to study the efficacy of different BTV vaccine compositions against challenge with different BTV serotypes. IFNAR(−/−) mice inoculated with DNA/rMVA-VP2,-VP5 in an heterologous prime boost vaccination strategy generated significant levels of neutralizing antibodies against BTV-4. This vaccine conferred partial protection against a lethal challenge with BTV-4. When VP7 was included into the vaccine composition, IFNAR(−/−) mice were completely protected against the homologous BTV-4 challenge (Calvo-Pinilla et al., 2009b). Immunodominant serotype cross-reactive T-cell determinants have been located within the

structural proteins of BTV-cores. VP7 is a major BTV group reactive antigen (Gumm and Newman, 1982) and sheep vaccinated with a capripox virus encoding VP7 showed clinical protection against heterotypic challenge, although the virus still replicated (Wade-Evans et al., 1996). These data led to define T cell epitopes in VP7. Rojas and colleagues identified novel CD4 and CD8T cell epitopes recognized in IFNAR(−/−) mice model from the VP7 core protein of BTV-8 while some of these epitopes were also recognized by T cells form infected sheep (Rojas et al., 2011).

Although the inclusion of VP7 improved the efficacy of the vaccine against an homologous challenge with BTV-4, this vaccine composition did not confer good protection against heterologous challenges with BTV-8 or BTV-1. For this reason, new compositions of antigens have been assayed in order to obtain a BTV vaccine that generates cross-protection against several BTV serotypes. When the protein NS1 of BTV-4 was included in the vaccine composition, in addition to VP2 and VP7, the heterologous prime boost vaccination strategy generated significant levels of antibodies specific of VP2, VP7, and NS1, including those with neutralizing activity against BTV-4 in the immunized IFNAR(−/−) mice. Furthermore, vaccination stimulated specific $CD8^+$ T cell responses against these three BTV proteins. Importantly, the vaccine combination expressing NS1, VP2 and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4 infection and induced cross-protection against lethal doses of heterologous BTV-8 and BTV-1, two serotypes that are not related phylogenetically, suggesting that the DNA/rMVA-VP2, -VP7, -NS1 marker vaccine is a promising multiserotype vaccine against BTV (Calvo-Pinilla et al., 2012). The importance of the inclusion of NS1 in the vaccine composition described in the IFNAR(−/−) mouse model has been confirmed later in cattle, a natural host of BTV. Anderson and colleagues observed measurable T cell responses against NS1 in cows immunized with VP2, NS1, and NS2, thereby supporting a bovine cross-reactive T cell response (Anderson et al., 2013).

Subsequent studies of vaccination using BTV-8 antigens showed that VP2 expressed in vivo using a heterologous or homologous prime boost vaccination (DNA/rMVA or rMVA/rMVA), can generate immunity against BTV-8 in IFNAR(−/−) mice, protecting them against a lethal challenge (Jabbar et al., 2013). The authors suggested that the differences observed in mice vaccinated against BTV-4 could be due to differences in immunogenicity between proteins of different serotypes. In addition, the dose of rMVA used in these studies was three times higher than the dose used in the studies of vaccination with BTV-4 antigens. This could also be a reason whereby total protection was observed when mice are immunized only with VP2.

8. Conclusions

IFNAR(−/−) mice are susceptible to the infection of BTV and the related orbivirus AHSV and EHDV. After infection, BTV infected mice show clinical signs characterized by ocular discharges, apathy and the disease progression led to animal death and infectious virus is recovered from the spleen, lung, thymus, lymph nodes and blood. Disease progression and pathogenesis closely mimics hallmarks of bluetongue disease in ruminants, and this mouse model of BTV infection is being used to study BTV transmission, pathogenesis, virulence, and protective efficacy of inactivated and new recombinant marker BTV vaccines. IFNAR(−/−) mice is a good choice to facilitate a faster advance in the field of orbivirus.

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Review

Recombinant vaccines against bluetongue virus

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ABSTRACT

Bluetongue (BT) is a hemorrhagic disease of ruminants caused by bluetongue virus (BTV), the prototype member of the genus Orbivirus within the family Reoviridae and is transmitted via biting midges of the genus Culicoides. BTV can be found on all continents except Antarctica, and up to 26 immunologically distinct BTV serotypes have been identified. Live attenuated and inactivated BTV vaccines have been used over the years with different degrees of success. The multiple outbreaks of BTV in Mediterranean Europe in the last two decades and the incursion of BTV-8 in Northern Europe in 2008 has re-stimulated the interest to develop improved vaccination strategies against BTV. In particular, safer, cross-reactive, more efficacious vaccines with differential diagnostic capability have been pursued by multiple BTV research groups and vaccine manufacturers. A wide variety of recombinant BTV vaccine prototypes have been investigated, ranging from baculovirus-expressed sub-unit vaccines to the use of live viral vectors. This article gives a brief overview of all these modern approaches to develop vaccines against BTV including some recent unpublished data.

Contents

1. Introduction

Bluetongue virus (BTV) was first detected in 1900, when Merino sheep were imported into South Africa and became infected (Spreull, 1905) showing clinical signs of hemorrhagic disease. This virus belongs to the *Orbivirus* genus, within the family *Reoviridae* and has a double stranded RNA genome that encodes four non-structural (NS1–NS4) and seven structural proteins (VP1–VP7) (Mertens et al., 1984; Mertens, 1986). The genome segments are packaged within an icosahedral capsid, ~80 nm in diameter, composed of three concentric protein layers. The innermost 'subcore' shell is constructed from 12 decamers of VP3, surrounding the virus genome and viral transcriptase complexes, and provides a 'scaffold' for addition of 780 copies of VP7 (organized as 260 trimers) to form the core-surface layer. The addition of 60 trimers of VP2 and 120

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trimers of VP5 which form the outer capsid layer, complete the virion structure (Roy, 1989).

The efficient transmission of the virus by *Culicoides* midges in areas with suitable vector species results in a fast spread of the disease. The disease has been devastating for European cattle and sheep especially since 1998 after repeated BTV outbreaks caused by 6 of the 26 different serotypes (Darpel et al., 2007; Maan et al., 2012). This has resulted in a big impact on trade and agriculture, making very important the development of new safe and effective vaccines against the virus.

Modified live vaccines have long been used to control BT in sheep in southern Africa, and more recently in Corsica, the Balearic Islands and Italy (Savini et al., 2008). Although live attenuated vaccines appear to be effective for protection of individual susceptible animals against clinical signs of bluetongue, they are teratogenic and cause other adverse effects (IZSAM, 2001; Veronesi et al., 2010). Moreover, the vaccine virus can be detected in blood after vaccination and reaches titers that are compatible with transmission to other mammalian hosts via *Culicoides* midges (Elia et al., 2008). When vaccine virus is transmitted to unvaccinated animals (Ferrari et al., 2005; Savini et al., 2008) there is a further risk of genome segment re-assortment between vaccine and field strains, leading to the emergence of new strains with unpredictable biological characteristics (Batten et al., 2008).

Whole inactivated virus vaccines represent a safer alternative to live attenuated vaccines and are commercially available but some concerns exist over the reliability of inactivation for each vaccine batch (Gethmann et al., 2009). Inactivated BTV vaccines have prevented the re-emergence of the disease in northern Europe in the years that followed the 2008 outbreak, however the cost of production of inactivated vaccines is high and various boosters are necessary to achieve solid immunity.

Serological screening techniques are essential for the surveillance of the disease in non-endemic countries when a vaccination campaign is followed and to demonstrate freedom of the disease once the outbreak has ended. There is currently not a reliable serological test to "distinguish infected from vaccinated animals" (DIVA assays) when either inactivated or attenuated BTV vaccines are used. For these reasons the development of recombinant BTV vaccines, which are inherently safer, and are based on selected BTV antigens and are therefore compatible with a DIVA approach, has been the subject of research over more than two decades. This review describes novel advances in the development of recombinant vaccines against BTV and the different studies testing their protective efficacy.

2. New-generation vaccines against BTV

Different strategies have been followed over the last 3 decades to develop novel recombinant vaccines for BTV, ranging from baculovirus expressed sub-unit vaccines to live virus vector vaccines (Table 1).

Immunological studies to date have given many clues about what BTV proteins are more important to induce protective host immune responses against the virus. The cellular receptor binding protein VP2 induces serotype specific neutralizing antibodies (Huismans and Erasmus, 1981) and protective immunity (Huismans et al., 1987). Epitope mapping studies revealed that the major virus neutralizing epitopes are located in the protein VP2 (Roy, 1992). This protein is the most variable protein among BTV serotypes and is the determinant of the serotype (Mertens et al., 1989). Antibodies raised against VP2 can sometimes neutralize, although less efficiently, other very closely relates serotypes (Roy, 1992). However 26 serotypes of BTV have been reported (Maan et al., 2012) and low levels of cross-protection are described, complicating vaccination strategies. VP5 protein is the second most variable of the BTV proteins and seems to exert some influence in the conformation of VP2 (Cowley and Gorman, 1989; Mertens et al., 1989). It has been demonstrated that both virus neutralizing antibody responses (Jeggo et al., 1984b) and cytotoxic T-lymphocytes (CTL) have a main role in protective immunity against BTV (Jeggo and Wardley, 1982; Jeggo et al., 1984a). BTV-specific CTL have been studied in sheep, describing VP2 and NS1 as major CTL targets (Andrew et al., 1995). NS1 was recognized by CTL from all Merino sheep immunized with recombinant vaccinia viruses, while VP2, VP3, VP5 and VP7 were recognized by CTL from only some sheep (Janardhana et al., 1999). In mice, statistical analysis of the CTL responses indicated that non-structural protein peptides were the predominant source of homotypic and heterotypic CTL recognition (Jones et al., 1996). In mice, T-cell epitopes (CD4 and CD8) were identified in the major BTV group-reactive antigen VP7 and these epitopes were also recognized by cells from BTV infected sheep (Rojas et al., 2011).

In general, vaccine approaches developed against viral diseases are preferably tested in a natural host. However, constraints imposed by the high cost of performing experiments in bio-containment facilities for large animals have led to the establishment of a small animal model for BTV (Calvo-Pinilla et al., 2009a). This murine model based on adult IFNAR (−/−) mice facilitates the study of the BTV immune responses and the testing of new vaccines against bluetongue. IFNAR (−/−) mice are lack the β-subunit of the interferon α/β receptor and this model has been used successfully in various studies with orbiviruses (Calvo-Pinilla et al., 2010; Castillo-Olivares et al., 2011; Eschbaumer et al., 2012).

2.1. Subunit vaccines

Protein-based vaccines against BTV have been developed using single proteins or by combining various proteins in the same vaccine preparation. Huismans et al. (1987) isolated VP2 from purified BTV by chemical means using divalent salts. Vaccination of sheep with this protein induced virus neutralizing antibodies and protection against virulent homologous challenge (Huismans et al., 1987). Despite its efficacy, this strategy could not be developed further due to the large amounts of virus required to produce enough purified protein for use in a sub-unit vaccine formulation.

Table 1
Recombinant viral vector vaccines against BTV tested in IFNAR (−/−) mice.

Delivery viral vector	Proteins expressed	Protection homologous challenge	Protection heterologous challenge
Bovine herpes virus	VP2 BTV-8	Partial	Not analyzed
Equine herpes virus	VP2 BTV-8	No	Not analyzed
Equine herpes virus	VP2, VP5 BTV-8	Partial	Not analyzed
MVA virus	VP2, VP5 BTV-4	Partial	Not analyzed
MVA virus	VP2, VP5, VP7 BTV-4	Complete	No
MVA virus	VP2, VP5, VP7 BTV-4	Complete	Complete
MVA virus	VP7, NS1 BTV-4	Partial	Partial
MVA virus	VP2, VP5, VP7 BTV-8	Complete	Not analyzed
MVA virus	VP2 BTV-8	Complete	Not analyzed

Following Huismans findings, Roy and co-workers developed a sub-unit vaccination strategy based on the expression of BTV proteins using recombinant baculovirus systems (Roy, 1990). BTV proteins derived individually from baculovirus vectors were administered in different combination and doses to sheep and protection was evaluated against homologous challenge with virulent BTV-10 (Roy et al., 1990). A minimal dose of 100 μg of VP2 was needed to protect sheep against challenge. However, when used in combination with 20 μg of VP5, a lower dose of VP2 (50 μg) was sufficient to elicit equivalent virus neutralizing antibody titers and to protect immunized sheep against BTV infection. This increased response was thought to be due to the synergistic effect of the two proteins. In these studies, the addition of VP1, VP3, VP6, VP7, NS1, NS2 and NS3 proteins to the VP2/VP5 based vaccines did not enhance the neutralizing antibody response but also protected all sheep.

The most successful subunit vaccines against BTV are based in the co-expression of the four major structural proteins of the virus (VP2, VP5, VP3 and VP7), which results in their assembly into "virus like particles" (VLPs). VLPs were first developed in 1990 for BTV-10 (Roy, 1990) and since then this approach has been used to produce VLPs from a variety of BTV serotypes. VLPs from BTV-10 or BTV-17 or from five serotypes in combination (BTV-1, BTV-2, BTV-10, BTV-13 and BTV-17) were tested in vaccination studies and raised specific neutralizing antibodies and a protective response in sheep against homologous challenge (Roy et al., 1994). Neutralizing antibody titers in animals that received doses of 50 μg of VLPs were not significantly higher than those vaccinated with 10 μg. This work also demonstrated that vaccination with VLPs from BTV-10 and BTV-17 induced neutralizing antibodies against the related (based on the amino-acid sequence of VP2) BTV-4, and partial protection was afforded against this heterologous serotype (Roy et al., 1994). Later on, another study described that again VLPs from BTV-8 were able to afford complete protection against a homologous viral challenge in sheep (Stewart et al., 2013). This approach was also effective in combination with VLPs from BTV-1 or BTV-2. Additional studies described homologous protection in sheep using VLPs from BTV-1 and interestingly in this case the protection was afforded against a virulent virus from a different geographic origin (Stewart et al., 2012).

Summarizing all these experiments with VLPs, a dose of BTV VLPs of 10 μg in combination with adjuvant afforded protection against virulent virus challenge. Both incomplete Freunds' adjuvant and an incomplete Montanide ISA-50 adjuvant proved to increase the immune response against BTV VLPs (Roy et al., 1992). Much lower doses of VP2 antigen were needed when VP2 was presented in the context of a VLP vaccine than when used alone as a sub-unit vaccine. Thus, just 2.39 μg of VP2 contained in 10 μg of VLP was necessary to achieve complete protection against challenge, whereas 100 μg of single VP2 were needed to achieve the same effect (Roy et al., 1990; Roy, 2002). It was proposed that the presence of other capsid proteins in the VLP had an influence in the conformation and folding of VP2 and therefore the correct presentation of virus neutralizing epitopes of this protein. This hypothesis is consistent with the finding that 50 μg of VP2 is only protective if co-administered with 20 μg of VP5 (Roy et al., 1990).

Additional studies using baculovirus expressed antigens showed that co-expression of VP3 and VP7 proteins resulted in the generation of "core like particles" (CLPs). Vaccination studies with these CLPs however did not induce complete immunity against BTV (Stewart et al., 2012; Thuenemann et al., 2013) suggesting that VP2 and possibly VP5 are essential components of a potential sub-unit BTV vaccine.

VLPs of BTV have been also produced in plants recently using the cowpea mosaic virus and their use in a vaccination study produced no clinical manifestations in sheep after homologous challenge, although viremia was no evaluated (Thuenemann et al., 2013).

Bacterial and yeast expression systems to produce recombinant BTV proteins have also been developed for several authors (Gould et al., 1994; Martyn et al., 1994; Pathak et al., 2008). Jabbar and co-workers expressed VP2, VP5 and VP7 proteins of BTV-8 by bacterial expression system and were tested as subunit vaccine in IFNAR (−/−) mice (unpublished). In this study, VP2 protein, either as a complete protein or as three separate overlapping fragments; and full-length VP5 and VP7 were expressed and used in vaccination experiments. When IFNAR (−/−) mice were vaccinated with fragmented VP2 together with VP5 and VP7 (with Montanide ISA-50V adjuvant), no neutralizing antibodies were induced and no protection was achieved after infection with 100 pfu of BTV-8. However when mice were vaccinated with complete VP2 together with VP5 and VP7 (also with Montanide), partial protection was observed against BTV-8 challenge since clinical score, mortality rates (50%) and viremia were lower than in the unvaccinated control mice. Vaccinated animals with complete BTV proteins showed neutralizing antibody response, albeit at low levels. It was suggested that the insolubility of the proteins used in the experiment may have had an effect on the lack of complete efficacy of the vaccination.

2.2. DNA vaccines

DNA vaccines have advantages as safety, easy manufacturing, biological stability and cost effectiveness. In the case of BTV, this vaccination approach was tested in our laboratory and it provided partial protection when plasmids expressing VP2, VP7 and NS1 proteins from BTV-4 were used in combination to vaccinate IFNAR (−/−) mice (unpublished). Mice were vaccinated twice with 50 μg of each plasmid two weeks apart and challenged with 10^6 pfu of BTV-4. Although there was no clinical protection, viremia was delayed in DNA-BTV immunized animals in comparison with non-immunized animals.

Although DNA vaccines often have low immunogenicity, they can be useful to prime the immune system when used in heterologous vaccination regimes in combination with recombinant viruses as boosting agents. This heterologous vaccination strategy was successfully used in various studies (Calvo-Pinilla et al., 2009b, 2012; Jabbar et al., 2013).

2.3. Recombinant viral vector vaccines

These vaccines are based on recombinant viruses that carry genes encoding BTV antigens for their expression in the host upon inoculation. Immunity induced by recombinant viral vaccines is normally attributed to the capacity of the recombinant virus to express the gene of interest at high levels within cells of the host. The viral vectors used for this purpose are attenuated to the host and are therefore inherently safe and because they carry the transgenes of interest devoid of other molecular regulatory elements of the parental virus the risk of gene segment re-assortment with field bluetongue strains is highly reduced. Viruses such as canarypox, capripox, vaccinia and herpes virus have all been tested as gene delivery systems for BTV-vaccination with different levels of success (Boone et al., 2007; Calvo-Pinilla et al., 2009b; Franceschi et al., 2011; Ma et al., 2012; Savini et al., 2008). In general, vector vaccines expressing BTV VP2 have been the most successful, eliciting protective immune responses in animals against homologous challenge, which is consistent with the data obtained from protein based vaccines (Tables 1 and 2).

Table 2
Recombinant viral vector vaccines against BTV tested in sheep.

Delivery viral vector	Proteins expressed	Protection homologous challenge	Protection heterologous challenge
Capripox virus	VP7 BTV-1	Not analyzed	Partial
Capripox virus	VP2, VP7, NS1, NS3 BTV-2	Partial	Not analyzed
Canarypox virus	VP2, VP5	Complete	Not analyzed
Vaccinia virus WR	VP2	Partial	Not analyzed
Vaccinia virus WR	VP2, VP5	Partial	Not analyzed
MVA virus	VP2, VP7, NS1 BTV-4	Not analyzed	Partial

2.3.1. Herpes viruses

Herpes viruses have been used extensively in gene therapy applications since these large enveloped double stranded DNA viruses can accommodate large foreign gene inserts. Bovine and equine herpes viruses have been used to construct recombinant vectored vaccines against BTV-8 achieving partial protection in vaccination-challenge experiments in IFNAR (−/−) mice.

2.3.1.1. Bovine herpes virus. A non-pathogenic strain of bovine herpes virus 4 cloned as a bacterial artificial chromosome (BAC-BoHV-4-A) was used to generate a recombinant BoHV-4 expressing BTV-VP2. The protective efficacy of BoHV-4-VP2 was tested in the IFNAR (−/−) mouse model (Franceschi et al., 2011). In this study, 6 mice were vaccinated two weeks apart with 10^6 pfu BoHV-4-VP2 and virus neutralizing antibodies were detected at a 1:10 serum dilution in all mice and at a 1:20 serum dilution in 50% of the animals. Two weeks after second vaccination, mice were challenged with 10 pfu of BTV-8. Both clinical signs and viremia were delayed by 5 days in the immunized animals in comparison with the control mice. One vaccinated mouse remained alive until the end of the experiment, indicating that the 17% of the vaccinated animals were completely protected. Therefore, the BoHV-4-VP2 was capable of elicited partial protection against homologous BTV in this mouse model.

2.3.1.2. Equine herpes virus type 1. In a more recent study, an equine herpes virus (EHV) was used as a vaccine delivery system of VP2 and VP5 from BTV-8 (Ma et al., 2012). The EHV-1 strain RacH has been established as an infectious bacterial artificial chromosome (BAC), making easy the manipulation of the virus genome. Live-vectored vaccines based on the RacH strain have been developed against several viruses and were shown to provide protection against disease since they induce both humoral and cellular immune responses (Rosas et al., 2007, 2008; Said et al., 2011). Two EHV-1 RacH-based recombinant vaccines expressing the immunodominant outer capsid protein VP2 of BTV-8, either alone (rH_VP2) or in combination with VP5 (rH_VP2_VP5) were evaluated in the IFNAR (−/−) mouse model against homologous challenge with 5×10^3 pfu of BTV-8. Immunization with two doses of 10^6 pfu of rH expressing VP2 alone did not protect mice against BTV-8 challenge. Although the course of the disease was slightly delayed in rH_VP2 vaccinated mice in comparison with the control group all vaccinated mice died by day 7 after challenge infection. In contrast, a higher degree of protection was observed when VP2 and VP5 were used in combination. Mice immunized twice with the rH_VP2_VP5 only transiently displayed mild disease (stilted gait, ruffled coat) and weight loss (about 5%), but fully recovered by day 9.

2.3.2. Poxviruses

Like herpesvirus vectors, poxviruses can accommodate large fragments of foreign DNA but poxvirus replication occurs within the cytoplasm of infected cells, eliminating the risk of virus persistence and genomic integration in host DNA. Various members of the family *Poxviridae* have been used as to express BTV antigens. The most widely used are capripox, canarypox and vaccinia viruses.

2.3.2.1. Capripox. A recombinant capripox virus (rCPV.BTV1.VP7) expressing VP7 from the BTV-1 South Africa strain (BTV-1SA) was generated by cloning the segment 7 of BTV-1SA into the plasmid pCR-3 under the control of the late vaccinia promoter p11. The capripox virus thymidine kinase (TK) DNA sequences flanking the BTV segment 7 in the plasmid enabled the integration of the expression cassette, via homologous recombination, into viral genome of the KS-1 capripox vaccine strain viral at the TK locus. In order to test the efficacy of this vectored vaccine, eight lambs were vaccinated with 1.5×10^7 pfu of rCPV.BTV1.VP7 and four lambs were vaccinated with the KS-1 vaccine strain. All animals vaccinated with rCPV.BTV1.VP7 showed a significant titer of antibodies against VP7 as measured by ELISA in contrast to animals vaccinated with the KS-1 vaccine strain. However, the virus neutralizing antibody titers were negative until challenge. All vaccinated animals and unvaccinated controls were challenged 5 weeks post-vaccination with a lethal dose of BTV-3. All KS-1 vaccinated and the non-vaccinated lambs died. Mortality of the rCPV.BTV1.VP7 vaccinates was reduced to 25%. The rest of vaccinated animals fully recovered at week four post-vaccination. It was proposed that the partial cross-protection observed was mediated by cell-mediated immunity against VP7 (Wade-Evans et al., 1996).

Additional vaccination studies have been carried out with other recombinant capripox viruses expressing VP2, VP7, NS1 and NS3 proteins of BTV-2 (Perrin et al., 2007). All those genes were cloned individually into pKSCATpSGPT under the early/late synthetic vaccinia promoter PS and recombinant capripox viruses were generated by homologous recombination of the expression cassette into the TK locus of the KS-1 capripox virus strain. Sheep were inoculated either with 2×10^6 $TCID_{50}$ of each recombinant capripox or a recombinant capripox expressing haemagglutinin of the peste des petits ruminants virus (PPRV) as control. Animals vaccinated with recombinant capripox expressing either NS3, VP7 or VP2 developed an antibody response against the transgenes. The NS1 capripox failed to do so. When animals were challenged with 10^4 $TCID_{50}$ of BTV-2, all vaccinated and non-vaccinated animals developed viremia. However there was a delay in the detection of BTV genome by RT-PCR in immunized sheep. One animal out of 11 died in the BTV-Cpox group whereas 3 animals out of 10 died in the negative control group. Herein capripox viruses expressing VP2, VP7, NS1 and NS3 proteins conferred partial protection in sheep against homologous challenge.

2.3.2.2. Canarypox. Recombinant canarypox expressing VP2 and VP5 proteins have also been tested as potential vaccines for BTV (Boone et al., 2007). Segments 2 and 5 from BTV-17 were cloned in the same plasmid transfer vector under two different poxvirus promoters flanked by the canarypox C5 DNA sequences which enabled the insertion of the dual expression cassette into the C5 locus of canarypox virus. Six sheep were vaccinated with the recombinant canarypox expressing VP2 and VP5 (BTV-CP) and 5 sheep were vaccinated with recombinant canarypox expressing proteins of West Nile virus (WNV-CP) as control. Prime boost vaccination induced specific BTV-17 neutralizing antibodies in BTV-CP group at week 4. After challenge with $10^{5.5}$ $TCID_{50}$ of BTV-17 at day 34, none of the vaccinated animals showed clinical signs or viremia, even 2 out of 6

animals did not seroconvert to VP7 after challenge, suggesting that the replication of the virus were not enough to produce antibodies against this protein. The control group showed clinical signs at 6 days after challenge and high viremia was detected.

2.3.2.3. Vaccinia virus. The first trial of Vaccinia virus as vaccine against BTV was in 1997, using a recombinant vaccinia virus WR strain, that expressed both VP2 and VP5 (VV-VP2-VP5) or VP2 alone (VV-VP2) of Australian BTV serotype 1, although this approach has not been pursued further. The vaccination induced variable titers of neutralizing antibody in sheep and afforded partial protection against homologous challenge with no detectable viremia in all sheep immunized with VV-VP2 and 66% of sheep vaccinated with VV-VP2-VP5 (Lobato et al., 1997).

2.3.2.4. Modified Vaccinia Ankara. Subsequent studies using Vaccinia as a recombinant viral vaccine vector were based on Modified Vaccinia Ankara (MVA). This virus was obtained from the Chorioallantois vaccinia virus Ankara which was passaged over 570 times in cell culture. As a result, MVA lost 15% of the parental genome and replication deficient in humans and most mammalian cells (Esteban, 2009; Mayr et al., 1978). Recombinant MVA (rMVA) expressing immunogenic viral proteins has been shown to induce both humoral and cell mediated immunity (Ramirez et al., 2000). This attenuated virus has a well-established safety record and history of use as a vaccine for infectious diseases (Esteban, 2009; Kennedy and Greenberg, 2009).

MVA has been used to construct many vectored vaccines expressing different proteins from different BTV serotypes. The transfer plasmid pSC11 has been used in all cases to clone the BTV genes of interest under the control of the vaccinia virus (VV) early/late promoter p7.5. Finally recombinant MVAs were generated after homologous recombination in cells between the TK gene sequences of pSC11 and those of wild type MVA virus. All of these recombinant vectors have been tested in IFNAR (−/−) mice and very recently one of the approaches has been also tested in sheep.

Initial vaccination studies in IFNAR (−/−) mice with recombinant MVA virus (rMVA) expressing BTV-4 antigens were performed using a plasmid DNA (pcDNA3) prime-rMVA boost vaccination regime. This heterologous vaccination regime was chosen since DNA priming has been described to improve the expansion of specific primed immune cells. In these experiments the dose of each rMVA used in vaccination was 10^7 pfu per mouse and the dose of each DNA used was 50 μg per mouse (Calvo-Pinilla et al., 2009b; 2012).

Thus, IFNAR (−/−) mice inoculated with DNA-VP2-VP5 and boosted with rMVA-VP2-VP5 generated high levels of neutralizing antibodies against BTV-4. After a lethal challenge with 10^3 pfu/mouse of BTV-4, the appearance of viremia was delayed in vaccinated animals and their highest viremia levels were 10-fold lower than in infected non-immunized mice. Whilst all non-immunized animals died by day 4 post-challenge, 80% of the vaccinates showed a delay of around 48 h in the onset of death and 20% of them were protected. Therefore, this DNA/rMVA-VP2-VP5 vaccination conferred partial protection against a lethal challenge with BTV-4. In further studies, rMVA and cDNA expressing VP7 was included in the vaccination regime to improve the effectiveness of the vaccine. Similar levels of virus neutralizing antibodies were stimulated with this vaccination regime but in addition VP2 and VP7 specific BTV T-cell responses were detected in the vaccinated mice. In this study, whereas all unvaccinated animals died, mice vaccinated with DNA/rMVA-VP2-VP5-VP7 did not show clinical signs of disease or viremia at any point after infection with the virus, even by qRT PCR, so they were completely protected against lethal BTV-4 challenge (Calvo-Pinilla et al., 2009b). Although this vectored vaccine conferred sterile protection against homologous BTV-4 challenge, when this vaccine was evaluated against the heterologous serotype BTV-8, only a delay of the disease was observed and therefore no heterotypic protection was conferred against a different serotype.

In order to develop a BTV vaccine that elicits cross-protection against other BTV serotypes, NS1 expression vaccine vectors were tested (Calvo-Pinilla et al., 2012) since NS1 is one of the most conserved proteins amongst BTV serotypes (Barratt-Boyes et al., 1995). Previous studies to map the location of BTV epitopes recognized by CTLs showed that NS1 was recognized by CTL from sheep and mice (Janardhana et al., 1999; Jones et al., 1996). On the other hand, VP5 was no longer included in the vaccination regime because virus neutralizing antibody responses of animals vaccinated with VP2 and VP5 vectors were not significantly higher than those vaccinated with VP2 vaccine vectors. Moreover VP5 protein is highly variable amongst serotypes so it was assumed the protein is not going to contribute to the cross-protection. It is known that NS1 assembles into tubules (Hewat et al., 1992) and in our studies it was observed that co-expression of VP2, VP7 and NS1 in transfected BHK-21 cells generates aggregates containing the three BTV proteins (Calvo-Pinilla et al., 2012). In general, aggregates are protected from degradation, which allows them to remain intact for interaction with antigen-presenting cells and their internalization to appropriate MCH class II-loading compartments, effectively enhancing their presentation of T cells (De Temmerman et al., 2011; Rosenberg, 2006). Therefore for further vaccination studies in mice, DNA/rMVA-VP2-VP7-NS1 vectors were used to test protection against homologous BTV-4 or heterologous BTV-8 or BTV-1 challenges. Thus, all vaccinated mice were protected against clinical signs and viremia following a lethal BTV-4 challenge (even no BTV RNA was detectable by qRTPCR). Therefore DNA/rMVA-VP2-VP7-NS1 induced a complete sterile protection in vaccinated mice against homologous BTV-4.

For the heterologous challenges, mice were infected with 100 pfu of BTV-8 or BTV-1. All immunized animals were completely protected against lethal challenge with these heterologous serotypes. In contrast, all the non-immunized mice died after challenge with BTV-8 or BTV-1 between day 5 and 6 or between days 6 and 7 respectively (Fig. 1A). Moreover no infectious virus was detected in blood samples of immunized mice after infection with BTV-8 or BTV-1 at any time of the experiment (Fig. 1B). Overall these results show that cross-protection is achieved with DNA/rMVA-VP2-VP7-NS1 vaccination against other serotypes of BTV. In terms of immune response in the vaccinated animals, high levels of virus neutralizing antibodies against BTV-4, but not against BTV-8 or BTV-1, were detected, as expected (Fig. 1C). As well a specific T-cell response activation was seen in immunized animals, since VP2, VP7 and NS1 proteins induced the expression of IFN-γ by CD8+ T cells upon re-stimulation (Fig. 1 D) (Calvo-Pinilla et al., 2012).

To analyze whether the combination of the three antigens VP2, VP7 and NS1 from BTV-4 was essential for a complete protection against at least homologous challenge, DNA/rMVA-NS1, DNA/rMVA-VP2-VP7 and DNA/rMVA-VP7-NS1 were tested separately (unpublished data). Groups of mice were vaccinated with each construct and four weeks after first immunization animals were challenged with same lethal dose of BTV-4 as in previous experiments. After infection, a delay in the onset of clinical signs and lower viremia was observed in all groups of vaccinated mice in comparison with the control mice inoculated with empty DNA/MVA. Immunization with vectors expressing NS1 alone did not protect all vaccinated animals but protected 16% of the mice. In mice immunized with DNA/rMVA-VP7-NS1, protection was better and 25% of animals survived the challenge. Finally DNA/rMVA-VP2-VP7 vaccination increased the survival rate of vaccinated animals to 32%. These experiments confirmed these vectors expressing VP2,

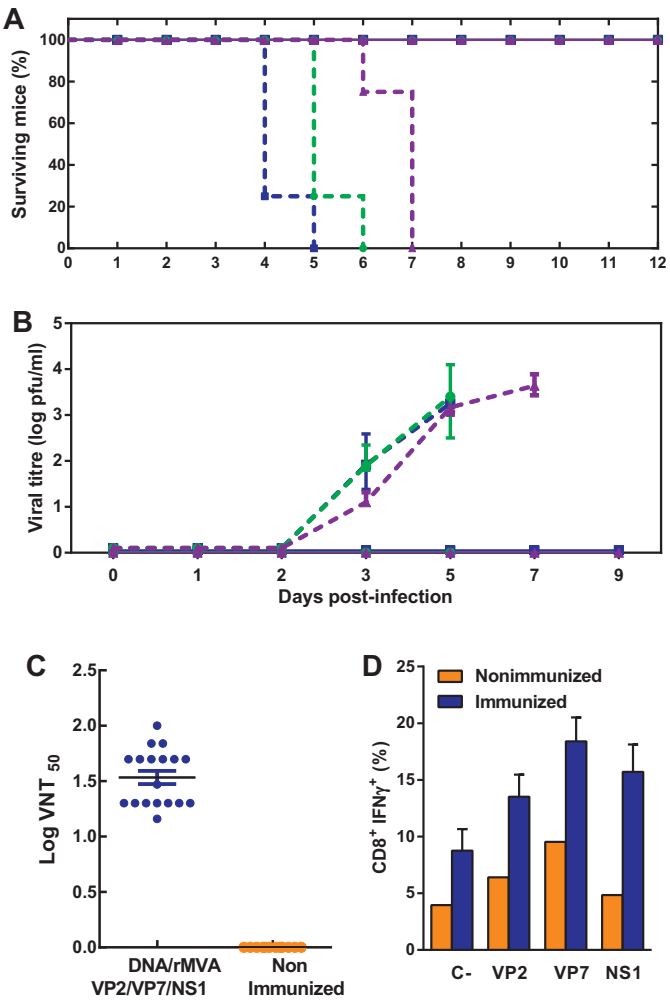

Fig. 1. Vaccination with DNA/rMVA-VP2-VP7-NS1 protects IFNAR (−/−) mice against homologous or heterologous BTV challenge. Six groups of six mice were vaccinated either with DNAs and rMVAs expressing BTV-4 proteins (continue lines) or with empty vectors (non-immunized, dotted lines). Two weeks after second vaccination mice were infected with 10^3 pfu of BTV-4 (blue), 100 pfu of BTV-8 (green) or 100 pfu of BTV-1 (purple). (A) Survival rates of the six groups of mice after challenge during 12 days. (B) Viral load means (log pfu/ml) in blood of mice at different times post-infection. SD are shown as error bars. (C) BTV-4 neutralizing antibodies titer in immunized (blue) and non-immunized mice (orange). (D) Intracellular staining of IFN-γ, in T CD8+ cells of immunized and non-vaccinated mice. Two weeks after second immunization spleens were harvested and splenocytes were stimulated with 10 μg/ml of VP2, VP7 or NS1 proteins. Error bars show SD.

VP7 and NS1 were necessary in combination for a total protection against BTV-4 challenge. Interestingly, when DNA/rMVA-VP7-NS1 was tested against heterologous BTV-8 challenge, a survival rate of 25% was achieved for the vaccinated animals and then the heterotypic protection achieved was exactly the same as with the homologous challenge (unpublished data). Host immune responses induced by VP7 and NS1 BTV-4 proteins had equal protective effect to protect animals against the same or different serotype, demonstrating the cross-protection properties of these proteins. Protective immunity against BTV is traditionally attributed to the induction of virus neutralizing antibodies which was not generated following vaccination with DNA/rMVA-VP7-NS1. These results suggested that cellular immune responses against VP7 and NS1 proteins could play a role in virus clearance from the host.

In order evaluate the cross-protection induced by the VP2-VP7-NS1 vaccination strategy in a natural host, Marroquina lambs were immunized with DNA-VP2-VP7-NS1 (300 μg/lamb) and rMVA-VP2-VP7-NS1 (3×10^7 pfu/lamb) from BTV-4 and challenged with $10^{6.2}$ $TCID_{50}$ of BTV-8 two weeks after the second immunization. Viremia was analyzed and qRT-PCR data showed that vaccinated animals presented lower levels of viremia than non-immunized animals. White blood cell (WBC) count in non-immunized lambs was at its lowest level on day 5 post-challenge coinciding with the highest virus titers in blood. This is consistent with previous studies showing BTV infection causes leukopenia in ruminants (Foster et al., 1991). In contrast, immunized animals maintained normal levels (7×10^9 cells/L) at day 5 post-infection indicating that vaccinates were partially protected against BTV-8 infection. The absence of BTV-8-specific neutralizing antibodies in the immunized sheep, suggested that the cell-mediated immunity induced by the vaccination contributed to the partial heterotypic protection observed. This aspect will be analyzed in sheep as it has been already analyzed in the IFNAR (−/−) mice model.

Since the massive BTV-8 outbreak in Northern Europe occurred, potential MVA vaccines based on proteins from this serotype were constructed and tested. In studies by Jabbar and co-workers, the efficacy of homologous rMVA/rMVA and heterologous DNA/rMVA prime-boost vaccination regimes was compared in the IFNAR (−/−) mouse model. These studies used vaccine constructs expressing either VP2 alone, VP7 alone or a combination of VP2, VP5 and VP7 from BTV-8 (Jabbar et al., 2013). Although immunization with vectors expressing VP7 alone did not protect IFNAR (−/−) mice against BTV-8 challenge, vaccinated animals showed a delayed onset of clinical signs and the survival time was slightly longer than in the non-vaccinated mice. Moreover the study showed that both rMVA/rMVA or DNA/rMVA prime boost vaccinations, expressing either BTV-8 VP2 alone or a combination the three proteins, induced protective immunity against BTV-8 challenge in mice. Very similar levels of efficacy of both vaccination regimes were shown. Interestingly, complete protection was achieved with vaccine vectors expressingVP2 alone. The two groups of mice vaccinated with BTV-8 VP2 alone, using either a heterologous DNA/rMVA or homologous rMVA/rMVA strategy, were completely protected against clinical signs of BTV infection, and all of them survived the challenge (Fig. 2A) and had no detectable viremia by plaque assay (Fig. 2B). Only low levels of BTV RNA were detected in some individuals by qRT-PCR, as well as in mice vaccinated with the three proteins. Herein, these DNA and MVA vaccines expressing VP5 and VP7 did not improve the protection induced by BTV-8 VP2 alone. The additional expression of more BTV proteins was necessary to protect animals in the experiments described earlier, but in this case the protein VP2 was expressed from BTV-8, indicating that there could be differences in immunogenicity between same proteins of different serotypes. These results are consistent with other vaccination studies performed with another orbivirus, African horse sickness virus (AHSV), which showed that vaccination with a recombinant MVA expressing the outer capsid protein VP2 of AHSV protected IFNAR (−/−) mice against virulent AHSV (Castillo-Olivares et al., 2011). In other studies, recombinant viruses expressing BTV-8 VP2 alone were not enough to confer protection against challenge in mice; nevertheless the viral vectors used in those studies were different from MVA. It is still not clear why in some circumstances VP2 alone is enough to induce protective immunity but it is known that some antigens appear to elicit better or worst immune response using different delivery system.

2.4. Use of reverse genetics for vaccine development

2.4.1. DISC (Disabled-Infectious-Single-Cycle) vaccines

Another approach which is also compatible with DIVA diagnostics is the development of disabled infectious single cycle vaccines (DISC). These vaccines are based on the production of

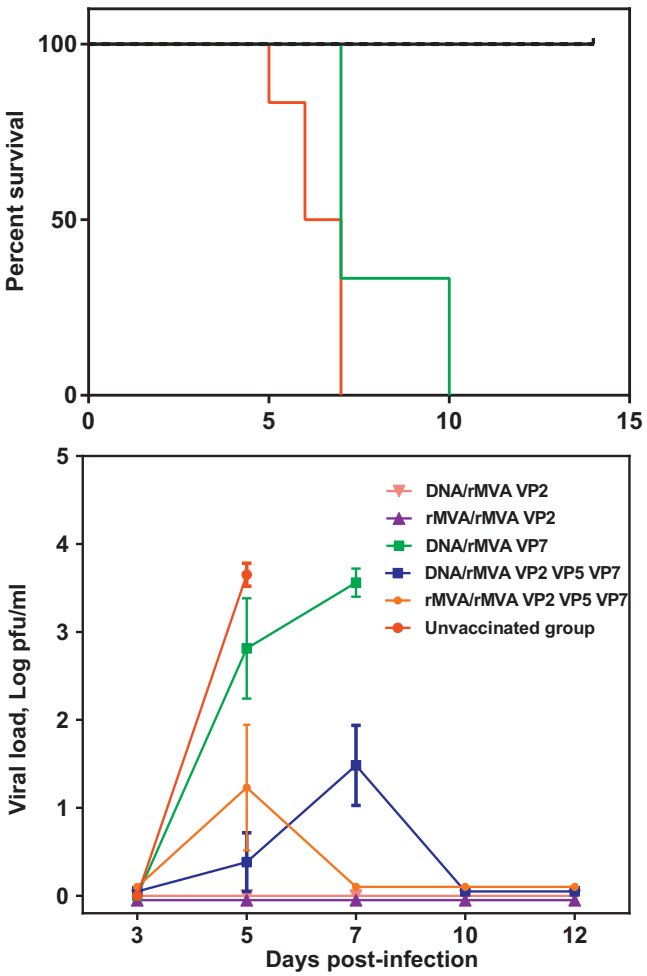

Fig. 2. Analysis of protection against BTV-8 after vaccination with DNA/rMVA or rMVA/rMVA vectors expressing VP2, VP5 and VP7 proteins, VP7 alone or VP2 alone. Groups of six mice were vaccinated with either DNA/rMVA or rMVA/rMVA expressing VP2, VP5 and VP7, VP7 alone or VP2 alone, and were challenged with BTV-8 five weeks after first immunization. (A) Percentage of survivorship after challenge of unvaccinated mice (red line), DNA/rMVA-VP7 (green line) and rest of the groups (black lines). (B) Viral load (log pfu/ml) of blood samples collected from vaccinated and control mice after challenge with BTV-8. Each point represents the mean values of the viral titer of six animals and standard errors are shown as bars.

modified virus with a deletion in one or more genes that are essential for virus replication. In cancer therapy, DISC-Herpes simplex virus encoding granulocyte macrophage colony stimulating factor (GM-CSF) has demonstrated its immunotherapeutic potential (Ali et al., 2002; Rees et al., 2002). Recently, a BTV reverse genetics technology to generate replication-deficient BTV serotypes has been developed (Boyce et al., 2008) making it possible the rescue of DISC viruses. This method is based on the introduction of a lethal mutation in one of the genes essential for replication, such as the gene encoding the viral helicase VP6 protein (Matsuo et al., 2011). A BTV-1 DISC virus was generated and used to construct a defective re-assortant by replacing the segments encoding for VP2 and VP5 with those from BTV-8 thereby creating BTV-1/8D1. Vaccination of sheep with these BTV-1 and BTV-1/8D1 DISC viruses prevented viraemia after homologous challenge with BTV-1 or BTV-8 respectively. Follow on studies showed that vaccination of sheep with a combination of DISC viruses induced a virus neutralizing antibody response against each of the serotypes represented in the vaccine (BTV-2, BTV-4 and BTV-8) and were protected against clinical signs and viraemia (Celma et al., 2013).

2.4.2. Gene segment re-assortant vaccines based on attenuated live BTV-6 virus

In a different use of reverse genetics, segments 2 (VP2) and 6 (VP5) of the live attenuated BTV-6 vaccine were exchanged for those of BTV-1 and BTV-8, resulting in the generation of BTVac-1 and BTVac-8, respectively (van Gennip et al., 2012). Vaccination of sheep with a single dose of these re-assortants induced a virus neutralizing antibody response against the homologous serotype. After challenge at three weeks post vaccination with cell-passaged virulent BTV-8, vaccinated animals showed nearly no clinical reaction. Furthermore, protection was similar between sheep vaccinated with BTVac-1 and animals vaccinated with BTVac-8. These data indicated that all sheep were partially protected from a challenge with homologous and heterologous serotype. However, virus genotyping analyses of blood samples from vaccinated sheep at 14 days post-challenge revealed the presence of BTVac-1, BTVac-8 and BTVac-6 but not the BTV-8 challenge virus suggesting a complete clearance of the challenge virus infection. These two examples of the use of reverse genetics showed the tremendous potential of this technology for vaccine development. Further work is needed to explore further the application of this strategy and also to address any potential bio-safety risk associated with the exchange of gene segments between vaccine and field viruses.

3. Conclusion

When conventional BTV vaccines were used in the past, serotype-specific control of bluetongue was achieved in response to specific outbreaks. However in order to overcome disadvantages of attenuated and inactivated vaccines, new effective recombinant vaccines have been developed over the last two decades. In addition recombinant vaccines expressing conserved protective antigens in order to generate an effective multivalent vaccine would reduce the number of multiserotype vaccinations required, therefore providing a cost-effective product. Despite the major advances in the understanding and prevention against BTV in trials with new generation vaccines, a commercial recombinant vaccine against this virus remains elusive. Usually animal health policy requirements for improved vaccines usually delays or precludes their implementation in the field. Important advantages of new generation vaccines comparing to conventional vaccines are the inherent safety and the possibility to distinguish serologically infected from vaccinated animals, which allow the control of the disease and surveillance. Right surveillance of a disease is highly important to authorize the safe movement of susceptible animals between affected and free zones. Moreover, experimental vaccines are useful tools to better understand the immune mechanisms activated in the animal to counteract the virus. For both reasons, development of recombinant vaccines against BTV is still essential nowadays.

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An experimental subunit vaccine based on Bluetongue virus 4 VP2 protein fused to an antigen-presenting cells single chain antibody elicits cellular and humoral immune responses in cattle, guinea pigs and IFNAR(−/−) mice

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ABSTRACT

Bluetongue virus (BTV), the causative agent of bluetongue disease (BT) in domestic and wild ruminants, is worldwide distributed. A total of 27 serotypes have been described so far, and several outbreaks have been reported. Vaccination is critical for controlling the spread of BTV. In the last years, subunit vaccines, viral vector vaccines and reverse genetic-based vaccines have emerged as new alternatives to conventional ones. In this study, we developed an experimental subunit vaccine against BTV4, with the benefit of targeting the recombinant protein to antigen-presenting cells. The VP2 protein from an Argentine BTV4 isolate was expressed alone or fused to the antigen presenting cell homing (APCH) molecule, in the baculovirus insect cell expression system. The immunogenicity of both proteins was evaluated in guinea pigs and cattle. Titers of specific neutralizing antibodies in guinea pigs and cattle immunized with VP2 or APCH-VP2 were high and similar to those induced by a conventional inactivated vaccine. The immunogenicity of recombinant proteins was further studied in the IFNAR(−/−) mouse model where the fusion of VP2 to APCH enhanced the cellular immune response and the neutralizing activity induced by VP2.

1. Introduction

Bluetongue virus (BTV) is the causative agent of bluetongue disease (BT) of domestic and wild ruminants. Among domestic species, sheep is the most susceptible and most severely affected host, but cattle and wild ruminants serve as reservoirs for the virus.

The BTV genome is composed of 10 segments of double-stranded RNA which encode for seven structural proteins (VP1–VP7) and five non-structural proteins (NS1, NS2, NS3/3a, and NS4) [1]. Among the structural proteins, VP2 is the most variable one, the determinant of serotype, responsible for hemagglutination, receptor binding and induction of serotype-specific neutralizing antibodies (NAs) [2].

Vaccination against BTV is an effective mean to control BT since it minimizes direct losses, reduces virus circulation and enables safe movement of animals [3]. Requirements for an ideal BTV vaccine include low cost, the possibility to use DIVA (differentiate infected and vaccinated animals) strategy, and induction of immunity against several serotypes. Both inactivated and attenuated vaccines are effective. However, there are concerns with regard to each; attenuated vaccines could cause teratogenic effects, decreased fertility and potential reassortment with reversion of the vaccine strain. Whereas inactivated vaccines represent a safer alternative, some concerns exist over incomplete inactivation and cost productions [3–8]. Regarding the delivery of BTV antigens, promising results have been obtained using poxvirus and other viral vectors [9–13]. In addition, subunit vaccines based on BTV virus-like particles (VLPs) or VP2, VP7, or NS1 proteins of BTV4 incorporated into avian reovirus muNS-Mi microspheres have been found to inhibit virus replication and disease signals after a challenge with the homologous virus [14–17]. Prime-boost vaccination

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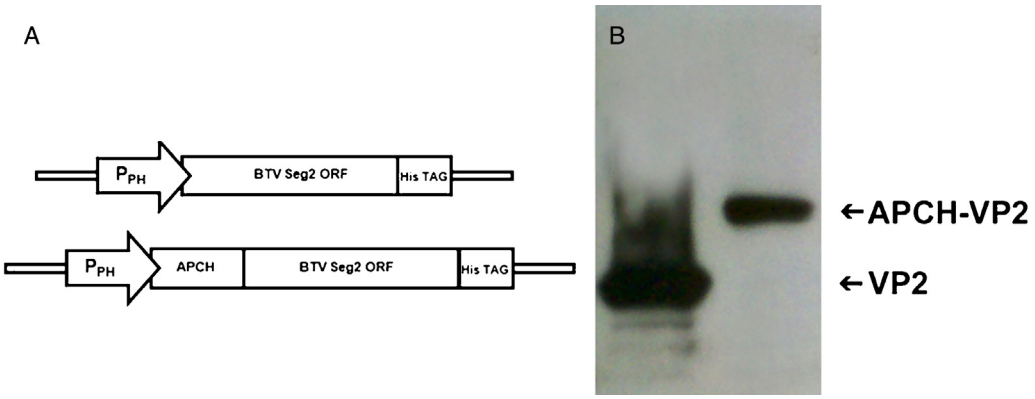

Fig. 1. pFastBac Dual schematic representation of the constructs for recombinant fusion protein expression (A). Production and detection of recombinant fusion proteins VP2 (110 kDa) and APCH-VP2 (140 kDa) in SF9 cells (B). Cell culture was infected at MOI 5 with recombinant baculoviruses. Infections progressed for 96 h for VP2 expression and 144 h for APCH-VP2 expression and total protein extracts were collected at the times indicated. Crude preparations were resolved by 8% SDS-PAGE and analyzed by Western blot using monoclonal antibody anti-Penta-His.

strategies have also been evaluated using naked DNA and recombinant modified vaccinia virus Ankara (rMVA) expressing BTV4 proteins [18,19]. In those studies, IFNAR(−/−) mice inoculated with DNA/rMVA expressing VP2, VP5, and VP7 generated a high level of NAs and complete protection against a homologous BTV4 challenge and the inclusion of NS1 in the DNA/rMVA vaccine composition conferred cross-protection against heterologous BTV1 and BTV8 challenges [18,19].

Subunit vaccines provide an opportunity to develop safe and rational vaccines, with the possibility of differentiating between vaccinated and infected animals. However, the challenge is to produce a vaccine capable of eliciting an efficient immune response with an affordable cost for veterinary applications. In this regard, considerable effort is being made to develop methods to enhance the immunogenicity of such vaccines. One of the most successful strategies under study is based on targeting the encoded antigens to specific sites of the immune cells. Antigen presenting cell homing 1 (APCH1), a single-chain variable fragment (scFv) that specifically recognizes an invariant epitope of the MHC II DR molecule on the surface of antigen-presenting cells, has been previously reported. This strategy has been demonstrated to be very efficient in improving the immune responses induced against many different antigens, using either recombinant subunit proteins or DNA vaccination [20–24].

In this work, the VP2 protein from an Argentine BTV4 isolate was expressed either alone or fused to APCH, a molecule that targets antigen-presenting cells (APCH-VP2) in the baculovirus insect cell expression system. The immunogenicity of both proteins was evaluated in guinea pigs, IFNAR(−/−) mice, and cattle.

2. Materials and methods

2.1. Construction of recombinant baculoviruses

BTV serotype 4 (BTV4) strain used in this study was an Argentine isolated named 4/ARG/829/2001, described by Legisa [25,26]. Segment 2 (Seg2) open reading frame was cloned into pFastBacDual plasmid (Invitrogen). Two constructs were generated: Seg2 ORF alone (VP2) and Seg2 tagged in its 5′ terminus to APCH (APCH-VP2) (Fig. 1 A). Recombinant baculoviruses were generated by using Bac-to-Bac Baculovirus Expression System (Invitrogen), according to the method recommended by the manufacturer. Briefly, Bacmids were generated transforming DH10Bac *E. coli* competent cells with 1 ng of plasmid. Positive clones were selected by color detection in LB Agar plates containing antibiotics. Bacmids were characterized by sequencing. *Spodoptera frugifera* cells (SF9) were transfected using Cellfectin II reagent and 2 μg of Bacmid. Supernatant were harvested at 72 h post-infection (hpi). This first supernatant containing recombinant baculovirus (P1) was used to infect SF9 cells at MOI 0.1 to increase baculovirus stock titter (P2).

2.2. Protein production

Optimal infection conditions were assessed for each recombinant baculovirus. For VP2 expression, Sf9 cells were infected at a multiplicity of infection (MOI) of 5 and harvested 96 hpi. For APCH-VP2, Sf9 cells were infected at a MOI of 5 and harvested 144 hpi. Protein samples were analyzed by 8% SDS-PAGE and western immunoblot using monoclonal antibody anti-Penta-His (QIAGEN).

2.3. Vaccine formulations

Vaccines were formulated with oil adjuvant Montanide ISA50 in a 60:40 adjuvant: antigen proportion. As positive control, BEI-inactivated BTV4 (4/ARG/829/2001) containing 8×10^6 $TCID_{50}$/ml was included. As a negative control, the same formulation was used with non-related recombinant baculovirus.

2.4. Immunization strategies

2.4.1. Immunization of guinea pigs

Eight- to 12-week-old guinea pigs (5 animals per group) were immunized either with (i) 0.2 or (ii) 2.4 μg of VP2 or with (iii) 0.15 or (iv) 0.6 μg of APCH-VP2, containing similar total protein mass. Guinea pigs were immunized on days 0 and 30. Immunogens were administered by the intramuscular route (i.m.). Sera were sampled at 0, 30 and 60 days post-inoculation (dpi).

2.4.2. Immunization of cattle

Aberdeen Angus cattle (6–8 months old) (5 animals per group) were used. Vaccine doses were: (i) 3.6 μg of VP2 and (ii) 0.9 μg of APCH-VP2. Groups were immunized on days 0 and 30 by i.m inoculation. Sera were collected at 0, 30, 60 and 90 dpi. Vaccine safety was evaluated throughout the assay.

2.4.3. Immunization of IFNAR(−/−) mice

In context of the IFNAR(−/−) animal model, eight-week-old IFNα/β$^{-/-}$ 129/sv mice (IFNAR(−/−) were used. Groups of five IFNAR(−/−) mice were immunized by prime-boost vaccination

with 10 μg of each recombinant purified protein administered 3 weeks apart. Virus challenge was conducted at 35 dpi. Mice were subcutaneously inoculated with 10^3 PFUs of BTV4. Mice were bled before each immunization and virus challenge. Sera were tested for BTV4 NAs by the standard virus neutralization Test (VNT) [27].

2.5. Animal welfare

Guinea pigs and cattle handling, inoculation, and sample collection were performed by trained personnel under the supervision of a veterinarian and in accordance to protocols approved by the Ethical Committee of Animal Welfare of INTA (CICUAE 20/2010, 40/2013 for guinea pigs assays and 45/2013 for cattle assays). Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 animal facilities at the CISA, INIA, Spain, for 1 week before use in our experiments. All experiments with IFNAR(−/−) mice were performed under the guidelines of the European community (86/609) and approved by the ethical review committee at CISA (Permit number: CEEA 2010-034). All efforts were made to minimize suffering.

2.6. Serum neutralization test

Serum NAs were detected by the virus neutralization assay [27]. Differences in antibody titers among experimental groups were evaluated by ANOVA under a model of repeated measures throughout time, followed by a general contrast post-ANOVA test. Statistical significance was assessed at $p < 0.05$ for all comparisons, using Statistix 8.

2.7. Competitive ELISA

Serum samples from 90 dpi were analyzed for anti-BTV antibodies, using C-ELISA kit BTV Antibody Test Kit (VMRD, Pullman, USA). This commercial kit detects anti VP7 antibodies.

2.8. Isotype-specific antibody ELISA

Specific IgG_1 and IgG_2 were detected by an indirect ELISA. Briefly, 96-well Plates 1B (Maxisorp, NUNC) were coated with 100 μl of cell culture supernatant from BHK21 cells either infected with BTV4 or non-infected. After each incubation period, three washes were made using PBS pH 7.4 Tween-20 (0.1%). The plate was blocked and samples were added in serial four-fold dilutions (1:8 to 1:128). Anti-IgG1 and anti-IgG2 monoclonal antibodies were added at 1:10,000 and 1:4000 dilutions, respectively. Horseradish peroxidase-conjugated anti-mouse serum was added and *O*-phenylenediamine–H_2O_2 was used as substrate. Absorbance was recorded at 492 nm.

2.9. Detection of epitope-specific $CD4^+$ and $CD8^+$ T cell responses by Intra-Cellular Cytokine Staining (ICCS)

Immunized mice were sacrificed 14 days post-booster and their spleens were harvested. ICCS was performed as described by Marín-López et al. [17]. Briefly, splenocytes from subunit vaccine-immunized mice were re-stimulated with 15 μg of recombinant BTV4 VP2 protein for 24 h and intracellular IFNγ production by $CD8^+$ T and $CD4^+$ T cells was determined by flow cytometry upon treatment with brefeldin A. Data were acquired by FACS analysis on a FACS Scalibur (Becton Dickinson) and analyzed with CellQuest Pro software.

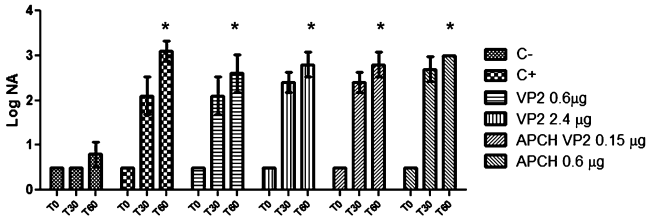

Fig. 2. Neutralizing antibody response to recombinant proteins VP2 and APCH-VP2 in guinea pigs. Animals were immunized with an oil adjuvant formulation of each protein. Each bar represents the geometric mean of NA titers of individual serum samples collected on day 0, 30 and 60 post-immunization. Error bars represent the standard error within the samples. Asterisks represent significant difference detected between T0 and T60 into each group (VP2; APCHVP2 or C+). At T30 and T60 significant differences were detected between negative control group and either subunit or BEI inactivated vaccines.

3. Results

3.1. Humoral response to the experimental subunit vaccine in guinea pigs and cattle

Seroconversion in guinea pigs was evident at 30 dpi in all immunized groups, while the negative control group remained negative throughout the experiment. After booster (60 dpv), animals immunized with VP2, APCH-VP2, or BEI-inactivated vaccine showed high NA titers which differed significantly from those at 0 dpv. There were no significant differences between the doses evaluated for each vaccine or between vaccines (Fig. 2).

Based on the results obtained in this experimental model, we next evaluated the immunogenicity of vaccines in cattle. Experimental groups included five animals each, which were immunized with either 3.6 μg of VP2 or 0.9 μg of APCH-VP2. The assay also included a positive control group (immunized with a BEI-inactivated BTV4) and a negative control group. At 30 dpi, after one immunization, specific NAs were detected in animals vaccinated with the recombinant proteins and with the inactivated vaccine (Fig. 3). Following the second vaccination, NA titers increased to very high levels in the vaccinated groups. In contrast, no NAs were detected in the control group at any of the time points analyzed (Fig. 3). After vaccination, none of the animals showed local reactions or adverse effects. It is important to note that the animals immunized with APCH-VP2 showed no significant differences with those immunized with either the VP2- or BEI-inactivated vaccines, although they received a four-fold lower dose of antigen.

To verify DIVA compliance of the experimental vaccines, a competition commercial ELISA was used to test cattle sera at 90 dpi. Animals immunized with BEI-inactivated BTV4 were positive for the test, while non-immunized animals and animals immunized with subunit vaccines were negative (data not shown).

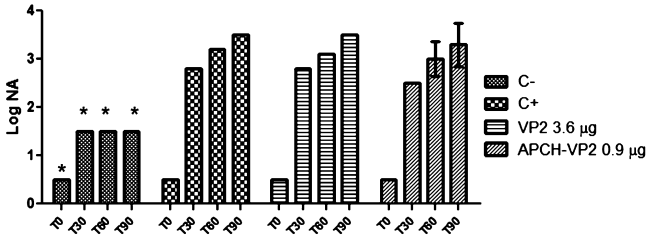

Fig. 3. Neutralizing antibody response to recombinant proteins VP2 and APCH-VP2 in cattle experiment. Animals were immunized with an oil adjuvant formulation of each protein. Each bar represents the arithmetic mean of NA titers of individual serum samples collected on day 0, 30, 60 and 90 post-immunization. Error bars represent the standard error within the samples. Asterisks represent significant difference detected between C- and treatments at T30, T60 and T90.

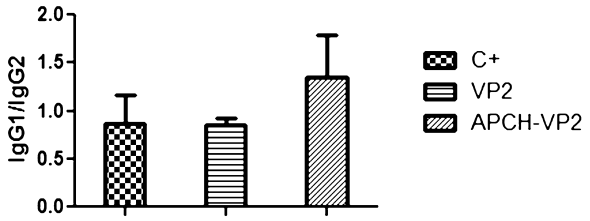

Fig. 4. Isotype-specific antibody ELISA. Specific IgG1/IgG2 ratio detected by an indirect ELISA. Serum samples from cattle at day 90 p.i. were analyzed and titer ratio were conducted. Bars represent the ratio between arithmetic means for titers measured by isotype ELISA in each treatment group.

3.2. IgG isotype profile induced by VP2 and APCH-VP2 in cattle

Sera from vaccinated cattle at 60 dpi were evaluated for the IgG isotype profile. IgG1 and IgG2 titers showed no significant differences both within and between vaccination treatments. However, the IgG1 titers tended to be higher than the IgG2 ones for APCH-VP2-vaccinated cattle (IgG1/IgG2 ratio near 1.4) (Fig. 4).

3.3. APCH-VP2 induces humoral and cellular immune response in IFNAR(−/−) mice

To evaluate the immunogenicity of the recombinant proteins VP2 and APCH-VP2, IFNAR(−/−) mice were inoculated intraperitoneally with 10 μg of each recombinant protein without adjuvant. Two weeks after the second immunization, immunized and control IFNAR(−/−) mice were challenged subcutaneously with 10^3 PFUs of BTV4.

Even when no adjuvant was used in the formulation of the vaccines, moderate NA response against BTV4 was elicited (Fig. 5A). Titers for APCH-VP2-immunized animals were higher than those for VP2-immunized animals.

To analyze whether APCH fused to VP2 improved the T-cell immune response elicited by VP2, the phenotype of the VP2 BTV4-specific IFNγ-producing T cells was analyzed by intracellular cytokine staining. Specific $CD4^+$ and $CD8^+$ T cells producing IFNγ following virus stimulation were observed in animals immunized with APCH VP2 meanwhile lower levels were recorded for animals immunized with VP2, showing that the cellular response in mice vaccinated with APCH-VP2 was enhanced (Fig. 5B and C).

4. Discussion

BT is included in the unified OIE list of notifiable terrestrial and aquatic animal diseases, then, major concerns are linked to viral presence and disease detection [28,29].

Historically, inactivated or attenuated vaccines have been used due to their low cost and easy production, despite the risk associated with their production. During the last decades, field isolates reporting reassortants comprising live vaccine strains, together with other biosafety issues, have raised concerns regarding the use of conventional vaccines and have encouraged the development of new generation vaccines.

The eradication of BTV from enzootic areas may not be easy, but, in non-enzootic areas, vaccination with inactivated vaccine incorporating the prevalent serotypes is advantageous [30]. However, inactivated vaccines are available in a few countries, including the European Union, India, the USA and China [30]. In this sense, development of recombinant vaccines in countries with the presence of only one or a few serotypes and with no local production of conventional vaccines becomes a promising strategy to combat BTV infection.

In this study, we developed an experimental subunit vaccine against BTV4 with the benefit of targeting the recombinant protein to antigen-presenting cells. In previous works, our group characterized Argentine isolates as a well-differentiated independent lineage [25]. However, the similarity analysis performed (data not shown) including amino acid sequences and comparing well-defined groups showed that the Argentine group and worldwide BTV4 isolates were highly similar and conserved the regions previously recorded as antigenic sites [31,32].

Huismans et al. first demonstrated in 1987 that VP2 was able to induce NAs and protection against homologous challenge in sheep [33]. After that, other reports in which VP2 was expressed using different expression systems have confirmed its role in protection [34–36].

In this study, high titers of specific NAs were induced in guinea pigs and cattle immunized with VP2 or APCH-VP2 expressed in the baculovirus system. Specifically, similar titers were reached for treatments including BEI-inactivated vaccine, VP2- and the APCH-VP2-based vaccines, although a four-fold lower antigenic mass was used in the APCH-VP2 group. The APCH molecule has been described as an immune response enhancer when it was fused to rabbit hemorrhagic disease virus, *Canine Parvovirus*, and Bovine viral diarrhea subunit vaccines [20,37,38]. In those reports, different expression systems as baculoviruses (sf9 cells and *Tricoplusia nii* larvae) or molecular farming (*Medicago sativa L.*) were used to express the recombinant antigens fused to APCH.

Recombinant vaccines comprising plasmid DNA or MVA virus encoding VP2, VP5 or VP7 proteins have been evaluated either alone or in combination. Whether the inclusion of VP5 and VP7 is critical for the induction of protection is still controversial. Some reports showed that complete protection is only achieved when VP2, VP5 and VP7 are used in combination in the vaccine composition [18,39,40]. However, other studies with BTV and other related

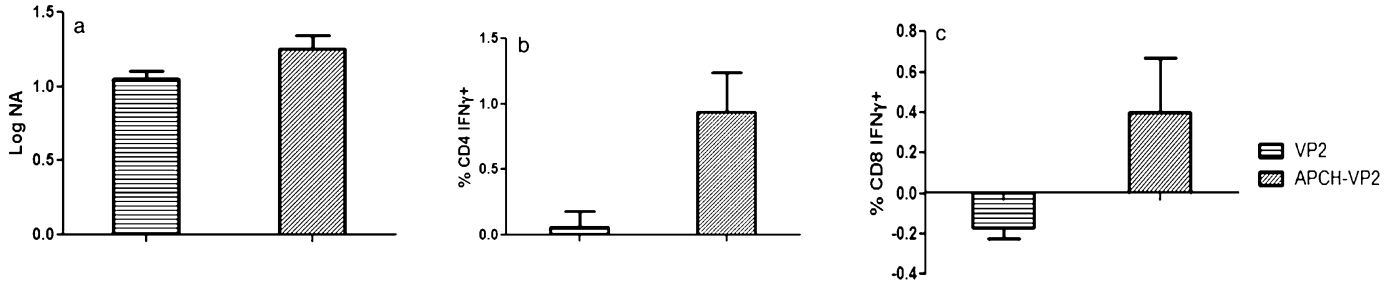

Fig. 5. IFNAR (−/−) experiments. (A) Humoral immune response observed in IFNAR(−/−) mice vaccinated with each vaccine. Specific neutralizing antibodies were analyzed in sera of immunized mice by VNT. Neutralization titers at day 14 post-booster treatment in sera of animals immunized with purified recombinant proteins means are presented as bars. (B and C) Cellular immune response detection of epitope-specific INFγ—CD4+ (B) and CD8+ T-cell (C) by Intra-cellular Cytokine Staining (ICCS). Specific Proliferation. were recorded in splenocytes obtained from mice immunized with recombinant proteins at 14 days post-booster. Splenocytes were stimulated in vitro with 15 μg of VP2 recombinant protein. The results represent the average of four mice after subtraction of background values ± SD.

orbivirus have shown that complete protection can also be achieved by subunit vaccines containing the VP2 protein alone [41–43].

Previous studies using VP2 as antigen have shown that a minimal dose of 100 μg VP2 is needed to protect sheep against the challenge; however, this dose could be decreased to 50 μg when VP2 is used in combination with VP5 [35,44]. Lower doses of VP2 were required when VP2 was present in the context of a virus-like particle. It has been reported that 10 μg of virus-like particles (containing 2.39 μg VP2) formulated with either incomplete Freund's adjuvant or incomplete Montanide ISA-50 adjuvant elicit protection against a virulent challenge [45]. In this work, we also observed that lower doses of APCH recombinant proteins, formulated with Montanide ISA-50 adjuvant, were needed to reach high antibodies titers in comparison with the recombinant protein alone. Specifically, a minimal dose of APCH-VP2 was needed in both guinea pigs and cattle (0.15 μg and 0.9 μg, respectively) to reach a specific antibody response similar to that obtained with VP2 (2.4 μg in guinea pigs and 3.6 μg in cattle) and the inactivated experimental vaccine. This result suggests that APCH could act as an effective enhancer for subunit vaccines and allow decreasing the antigen mass, which is a desirable feature for a subunit vaccine.

To characterize the immune response, an IgG isotype ELISA was conducted as a first approach. No significant differences between groups were found. However, the IgG1/IgG2 ratio showed a difference which suggests a special immune response pattern. The IgG1/IgG2 ratio obtained was similar to that of other reports using APCH fused to the E2t protein as a subunit vaccine for Bovine viral diarrhea virus [37]. These results could indicate that the APCH molecule slightly switches the isotype profile toward IgG1. This profile was also reported by Gil et al. [20]. This could be a desirable feature for an experimental vaccine used in the field since some studies have shown that colostrum containing NAs against BTV could protect against the virus infection [46,47].

In cattle, high NA titers were reached and no adverse effects were recorded. In addition, recombinant vaccines were tested to confirm their DIVA compliance. This DIVA feature of differentiate between infected and vaccinated animals is important, particularly in cattle, which are usually asymptomatic after BTV infection, but are able to spread the virus [44].

It has been reported that, in BT, the humoral response is the main component of the immune response against the virus and the disease. The cellular component is also important but, so far, how it works and how important its contribution is to the whole immune response are not well understood. To better understand the immunogenicity of both recombinant proteins, the IFNAR (−/−) mouse model was used to evaluate the immune response elicited by VP2 and APCH-VP2 proteins without adjuvant. Regarding the humoral response, moderate NA levels were recorded in animals vaccinated with APCH-VP2. Moreover, in the homologous challenge, a survival trend was recorded for both recombinant proteins (Supplementary Fig. 1). Since VP2 and APCH-VP2 were inoculated without adjuvant, a protective immune response was not expected. However, the net result of targeting the antigen to antigen-presenting cells could be observed without any masking effect of the adjuvant.

In the cellular immunity assays, APCH-VP2-vaccinated mice showed specific IFNγ $CD4^+$ and IFNγ $CD8^+$ cell proliferation, suggesting that APCH is an enhancer both of the humoral and cellular immune responses.

Summarizing, this work addressed the immunogenicity of a recombinant vaccine based on BTV4-VP2 protein fused to the molecule APCH, which enhances the immune response. As mentioned above, further experiments with APCH-VP2 should be performed to assess the effective dose capable of inducing the desired immune response. In addition, the T cell response in cattle should also be studied to understand the complete immune response induced.

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Conflict of interest statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2015.03.067

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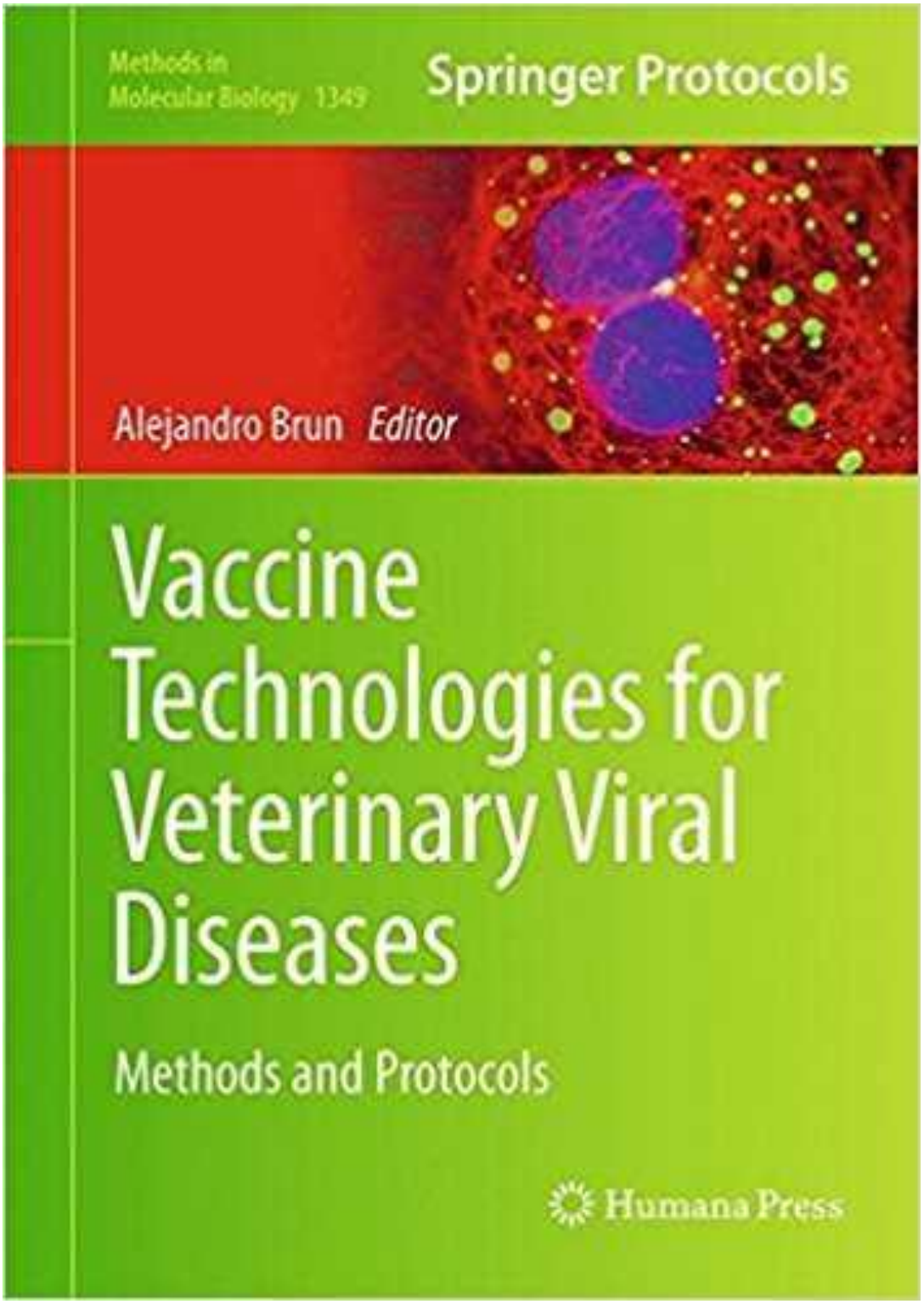
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Chapter 9

Generation of Recombinant Modified Vaccinia Virus Ankara Encoding VP2, NS1, and VP7 Proteins of Bluetongue Virus

Alejandro Marín-López and Javier Ortego

Abstract

Modified Vaccinia Virus Ankara (MVA) is employed widely as an experimental vaccine vector for its lack of replication in mammalian cells and high expression level of foreign/heterologous genes. Recombinant MVAs (rMVAs) are used as platforms for protein production as well as vectors to generate vaccines against a high number of infectious diseases and other pathologies. The portrait of the virus combines desirable elements such as high-level biological safety, the ability to activate appropriate innate immune mediators upon vaccination, and the capacity to deliver substantial amounts of heterologous antigens. Recombinant MVAs encoding proteins of bluetongue virus (BTV), an Orbivirus that infects domestic and wild ruminants transmitted by biting midges of the *Culicoides* species, are excellent vaccine candidates against this virus. In this chapter we describe the methods for the generation of rMVAs encoding VP2, NS1, and VP7 proteins of bluetongue virus as a model example for orbiviruses. The protocols included cover the cloning of VP2, NS1, and VP7 BTV-4 genes in a transfer plasmid, the construction of recombinant MVAs, the titration of virus working stocks and the protein expression analysis by immunofluorescence and radiolabeling of rMVA infected cells as well as virus purification.

Key words Recombinant modified vaccinia virus Ankara, Bluetongue virus, Viral-vectored vaccine, VP2, NS1 and VP7 proteins

1 Introduction

Vaccinia viruses engineered to express foreign genes are powerful vectors for production of recombinant proteins [1]. Modified vaccinia virus Ankara (MVA) was obtained from the Chorioallantois vaccinia virus Ankara (CVA) and was isolated following more than 500 passages in chick embryo fibroblasts. After this extensive propagation the viral genome suffered several major deletions and numerous small mutations resulting in replication defects in human and most other mammalian cells, as well as severe attenuation of pathogenicity [2–4]. This is why these viral vectors have shown

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excellent safety profiles (the vector can be used at biosafety level 1), significant immunogenicity against foreign expressed antigens and ability to induce protective immune responses [5]. Poxviruses can accommodate large fragments of foreign DNA and their replication occurs within the cytoplasm of infected cells, eliminating the risk of virus persistence and genomic integration in host DNA [3]. MVA has intrinsic adjuvant capacities ant it is being widely investigated as a safe smallpox vaccine and as an expression vector to produce vaccines against other infectious diseases and cancer [4]. Recombinant MVA (rMVA) expressing immunogenic viral proteins has been shown to induce both humoral and cell mediated immunity [1, 6].

Poxviruses have the ability to induce the expression of type-I and II interferons and to express soluble receptors capable of interacting with host antiviral mechanisms. This antagonist expression is minimized owe to the deletions in the rMVA genome, which contributes to the immunogenicity of this viral-vector used as a vaccine. Type-I interferons may act as a link between the innate and adaptive immune system, including humoral and cellular responses [7, 8]. MVA has been used to construct many vectored vaccines expressing different proteins from different kind of orbiviruses [3]. The transfer plasmid pSC11 [9] was designed to place the genes of interest (in our case from *bluetongue virus*) under the control of the vaccinia virus (VV) early/late promoter p7.5. Finally, rMVAs were generated after homologous recombination in permissive cells between the TK gene sequences of pSC11 and those of wild type MVA. In our laboratory, all of these recombinant vectors have been tested as potential vaccines in IFNAR$^{(-/-)}$ mice [10–15]. We engineered rMVAs expressing VP2, NS1, and VP7 proteins from BTV-4. IFNAR$^{(-/-)}$ mice were inoculated with DNA-VP2,-NS1,-VP7/rMVA-VP2,-NS1,-VP7 in an heterologous prime boost vaccination strategy, generating significant levels of antibodies specific of VP2, NS1, and VP7, including those with neutralizing activity against BTV-4. The vaccine combination expressing VP2, NS1, and VP7 proteins of BTV-4, elicited sterile protection against a lethal dose of homologous BTV-4 infection and induced cross-protection against lethal doses of heterologous BTV-8 and BTV-1, suggesting that the DNA/rMVA-VP2,-NS1,-VP7 marker vaccine is a promising multiserotype vaccine candidate against BTV [14].

This work details the methodology applied to generate the rMVAs encoding the proteins VP2, VP7, and NS1 of BTV-4. In addition, the chapter describes the protocols to analyze the BTV protein expression in DF-1 cells infected with these rMVAs by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE.

2 Materials

1. BTV serotype 4 (SPA2004/01).
2. Modified vaccinia virus Ankara (MVA) (generously provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
3. Kidney epithelial cells extracted from an African green monkey, *Chlorocebus* sp. (Vero cells) (ATCC, Cat. No. CCL-81).
4. Chicken embryo fibroblast (DF-1 cells) (ATCC, Cat. No. CRL-12203).
5. Serum and antibiotic free Dulbecco's modified Eagle's medium.
6. DMEM with 2 mM glutamine, 10 % fetal calf serum (FCS), and 1 % Penicillin/Streptomycin (complete DMEM).
7. TRI Reagent Solution (Ambion).
8. 10× RT buffer, 25 mM $MgCl_2$, 0.1 M DTT (Life Technologies).
9. SuperScript® III Reverse Transcriptase (200 U/μL) (Life Technologies).
10. RNaseOUT™ (40 U/μL) (Life Technologies).
11. 10× PCR Buffer II, 10 mM dNTPs, specific primer (VS and RS) (Table 1) (Life Technologies).
12. AmpliTaq DNA Polymerase (1.25 U/50 μL) (Life Technologies).
13. 1 % agarose gel (Tris–acetate–EDTA buffer (TAE) and 1 % agarose).
14. Midori green DNA stain (Nippon Genetics Europe GmbH).

Table 1

Primer used for amplification of BTV genes

Gene		Sequence
VP2	VS	5′-CGCCCGGGATGAACTAGGCATCCCAG-3′
	RS	5′-CGCCCGGGCATACGTTGAGAAGTTTTGTTA-3′
NS1	VS	5′-CGCCCGGGATGGAGCGCTTTTTGAGAAAATAC-3′
	RS	5′-CGCCCGGGCTAATACTCCATCCACATCTG-3′
VP5	VS	5′-CGCCCGGGATGGGTAAAGTCATACGATC-3′
	RS	5′-CGCCCGGGTCAAGCATTTCGTAAGAAGAG-3′
VP7	VS	5′-CGCCCGGGATGGACACTATCGTCGCAAG-3′
	RS	5′-CGCCCGGGCTACACATAGCGCGCGCGTGC-3′

*Sma*I restriction site *underlined*

15. Qiaex II Gel Extraction Kit (Qiagen).
16. pSC11 plasmid (kindly provided by Professor Francisco Rodríguez, Centro Nacional de Biotecnología, CSIC, Madrid, Spain).
17. *Sma*I restriction endonuclease, shrimp alkaline phosphatase (SAP), and T4 ligase enzyme.
18. Luria-Bertani (LB) agar plates and media.
19. Ampicillin sodium salt.
20. QIAprep® Spin Miniprep Kit (Qiagen).
21. Lipofectamine® Reagent (Invitrogen).
22. Noble agar (Difco Noble Agar (DB) and distilled water).
23. X-Gal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside).
24. Complete DMEM-0.6 % Noble agar with X-Gal (0.4 μg/μL) (complete DMEM–agar–X-Gal).
25. Formaldehyde 10 %.
26. Crystal violet in 80 % methanol.
27. Acetone–methanol solution (40 %/60 %).
28. Phosphate buffered saline 1× (PBS 1×).
29. PBS 1× 20 % FCS (blocking solution).
30. Mouse polyclonal antibody against BTV-4.
31. Alexa Fluor® 594 goat anti-mouse IgG (H + L) (Invitrogen).
32. ProLong Gold antifade reagent (Life Technologies).
33. RIPA buffer: 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 0.5 % sodium deoxycholate, 1 % Triton X-100, protease inhibitors.
34. Methionine-free DMEM cell culture medium.
35. [^{35}S] Methionine (800 Ci/mmol). (Amersham).
36. Dynabeads® Protein G system (Life Technologies).
37. SDS-PAGE buffer: 0.125 M Tris–HCl, 4 % SDS, 20 % v/v glycerol, 0.2 M DTT, 0.02 % bromophenol blue, pH 6.8.
38. 36 % sucrose cushion and sucrose gradient.
39. SW 28 centrifuge tube (50 mL).

3 Methods

These methods describe the generation of the recombinants MVAs encoding BTV-4 VP2, VP7 and NS1 proteins, the screening of positive recombinants, the upgrowth and quantification of virus stock, the analysis of BTV protein expression by immunofluorescence assay and radiolabeling, immunoprecipitation and SDS-PAGE in

infected DF-1 cells as well as the purification of the rMVAs generated in DF-1 cells.

3.1 Cloning of VP2, NS1, and VP7 BTV-4 Genes for Generation of Recombinant MVAs

Segments 2, 5, and 7 corresponding to VP2, NS1, and VP7 proteins will be amplified from total RNA of BTV-4 infected cells. To generate the MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7, the restriction site *Sma*I, must be introduced into the 5′ and 3′ ends of the PCR products, unique restriction site contained into pSC11.

1. Infect confluent Vero monolayers in M24-well plates (1.67×10^4 cells/well) with BTV serotype 4 (BTV-4) with a multiplicity of infection (MOI) of 1.
2. After virus adsorption for 1.5 h at 37 °C, 5 % CO_2, remove the medium, add 1 mL of complete DMEM and incubate for 24 h at 37 °C.
3. At 24 h post infection (h.p.i), when a clear cytopathic effect is observed, remove the supernatant and extract total RNA from infected cells with TRI Reagent Solution, according to the method recommended by the manufacturer (*see* **Note 1**).
4. The recovered RNA can be stored at −80 °C in small aliquots for later processing. RNA stored at this temperature is stable for prolonged periods of time (over 1 year).
5. Denature 5 μg of RNA in presence of 1 μL of 2 μM Reverse Sense (RS) BTV gene-specific primer (Table 1), 1 μL of 10 mM dNTP mix in a final volume of 10 μL by heating to 65 °C for 5 min and then rapidly cool on ice.
6. Add 2 μL 10× RT buffer, 4 μL 25 mM $MgCl_2$, 2 μL of 0.1 M DTT, 1 μL RNaseOUT™ (40 U/μL), and 1 μL SuperScript® III Reverse Transcriptase (200 U/μL).
7. Incubate the reaction for 1 h at 50 °C. Then, inactivate the reverse transcriptase by heating at 85 °C for 5 min. Chill on ice.
8. The cDNA produced is stable at 4 °C for short term storage, −20 °C for prolonged storage or used for Polymerase Chain Reaction (PCR) immediately.
9. Amplify the VP2, NS1, and VP7 cDNAs by PCR. Use 10 μL of 10× PCR Buffer II, 2 μL of 10 mM dNTPs, 2 μL of each specific primer (VS and RS) including *Sma*I site (Table 1), 4 μL of 25 mM $MgCl_2$ solution, 0.6 μL of AmpliTaq DNA Polymerase (1.25 U/50 μL), and 5 μL of cDNA template in a final volume of 100 μL.
10. Amplification cycle parameters are: 94 °C for 2 min (1×); 94 °C for 45 s, 55 °C for 1 min, and 72 °C for 2 min (30×); 94 °C for 15 min (1×).

11. Analyze the PCR products on a 1 % agarose gel stained with Midori green DNA stain (or other intercalating dye) and purify the PCR products with Qiaex II Gel Extraction Kit.
12. Digest the plasmid pSC11 and the purified PCR products VP2, NS1, and VP7 (containing the restriction site *Sma*I into the 5′ and 3′ ends) with the restriction enzyme *Sma*I as per manufacturer instructions.
13. Proceed to dephosphorylation of digested pSC11 with shrimp alkaline phosphatase (SAP) according to the method recommended by the manufacturer in order to prevent the plasmid self-ligation.
14. Purify the digested PCR products and the digested and dephosphorylated plasmid with Qiaex II Gel Extraction Kit.
15. Ligate the purified digested VP2, NS1, and VP7 with the purified digested plasmid pSC11 with T4 ligase enzyme according to the manufacturer's instructions. Perform the ligation at 16 °C overnight with a molar ratio of vector to insert of 1:3.
16. Transform the ligation products into chemically competent DH10B bacterial cells and plate out the transformants on LB agar with selection in the presence of ampicillin (100 μg/mL).
17. The next day select single colonies and grow in LB with ampicillin. Isolate the plasmid following the QIAprep Miniprep Handbook and analyze the presence and right orientation of the cloned VP2, NS1, and VP7 genes into the transfer plasmid pSC11 by sequencing (*see* **Note 2**).

3.2 Construction of Recombinant MVAs

The next step of the procedure is the generation of recombinant MVAs (*see* Fig. 1). The MVA transfer plasmids pSC11-VP2, pSC11-NS1, and pSC11-VP7 should contain the VP2, NS1, and VP7 BTV genes, flanked by thymidine kinase (TK) sequences of MVA, under the control of the vaccinia virus (VV) early/late promoter p7.5. Infection of cells with MVA and subsequent transfection with pSC-11 plasmids will generate recombinant viruses

3.2.1 Infection/Transfection of DF-1 Cells with MVA wt and pSC11 Plasmid Respectively

1. Plate DF-1 cells in p35 or six-well plates 1 day prior to infection in a 2 mL volume of complete DMEM.
2. DF-1 cells that are 60–80 % confluence are needed for infection and transfection.
3. Add 100 μL of MVA wild type (wt) in serum and antibiotic-free DMEM at 0.1 or 1 of MOI.
4. Incubate the cells at 37 °C and air–5 % CO_2 atmosphere for 1.5 h. After virus adsorption, DF-1 infected cells are transfected with pSC11-VP2, pSC11-NS1 or pSC11-VP7.
5. Mix 2 μg of plasmid in 50 μL of serum and antibiotic-free DMEM. Add to this mixture 9 μL of Lipofectamine® reagent

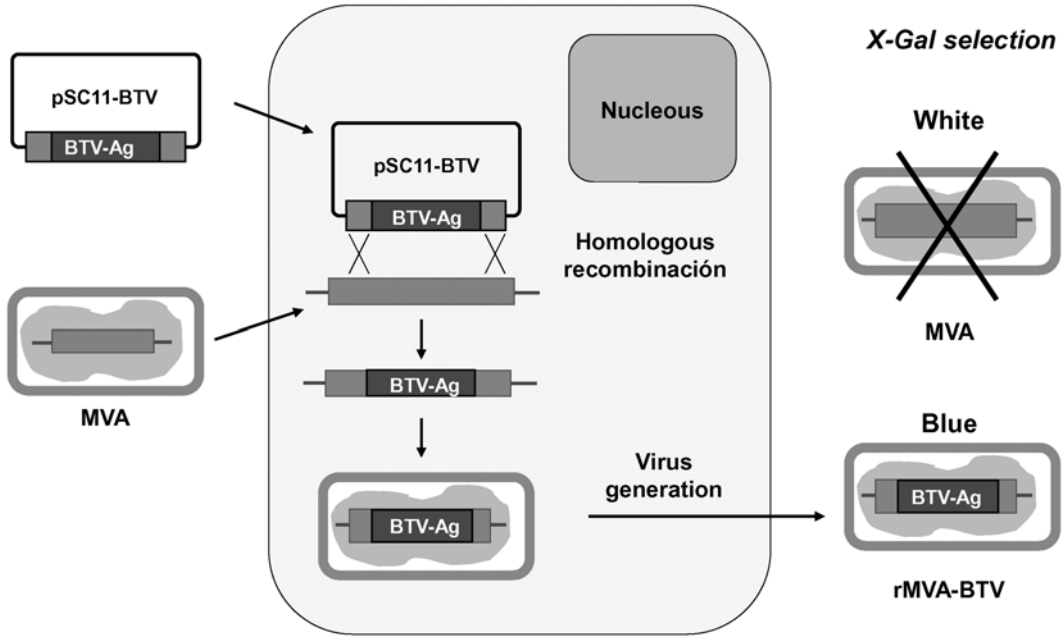

Fig. 1 General procedure for the generation of recombinant MVA. Genes VP2, NS1, and VP7 of BTV-4 were cloned into the vaccinia transfer plasmid pSC11 downstream of the p7.5 vaccinia promoter. DF-1 cells were infected with MVA virus (MOI 0.01 p.f.u./cell). After adsorption, cells were transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7 plasmids. Recombinant MVA viruses were generated by homologous recombination at the Thymidine kinase locus, allowing the analysis by using the LacZ marker

in 250 μL of serum and antibiotic-free DMEM and incubate at room temperature for 30 min.

6. Add 0.7 mL of serum and antibiotic-free DMEM to the lipid–plasmid complex. Add the total volume (≈1 mL) to the cells.
7. Incubate at 37 °C and air–5 % CO_2 atmosphere for 5 h, shaking the plate every 30 min.
8. Remove the lipid–plasmid complex and add 1 mL of complete DMEM.
9. Incubate at 37 °C and air–5 % CO_2 atmosphere for 72 h.
10. When the cytopathic effect (CPE) is apparent, harvest cells and supernatants by disruption of the monolayer (*see* **Note 3**). Carry out three cycles of thawing–freezing and sonicate twice for 10 s to disrupt the cells and release viruses.
11. Centrifuge at 2500 × *g* for 1 min. The supernatant will be used for the plaque analysis to look for recombinant MVAs as described below.

3.2.2 Plaque Purification of Recombinant MVA Viruses

In this step, we try to find clear, well-separated plaques for isolating and screening cloned viruses (*see* Fig. 2).

1. Plate DF-1 cells in six-well plates and incubate until they reach 80 % confluence.

2. Use the supernatants from the infected-transfected cells to do tenfold dilutions on the DF-1 cells from undiluted to 10^{-7} (*see* **Note 4**).
3. Allow the viruses to adsorb at 37 °C for 1 h.
4. Aspirate the supernatants and add 1.5 mL of complete DMEM.
5. Incubate at 37 °C and air–5 % CO_2 atmosphere for 72 h.

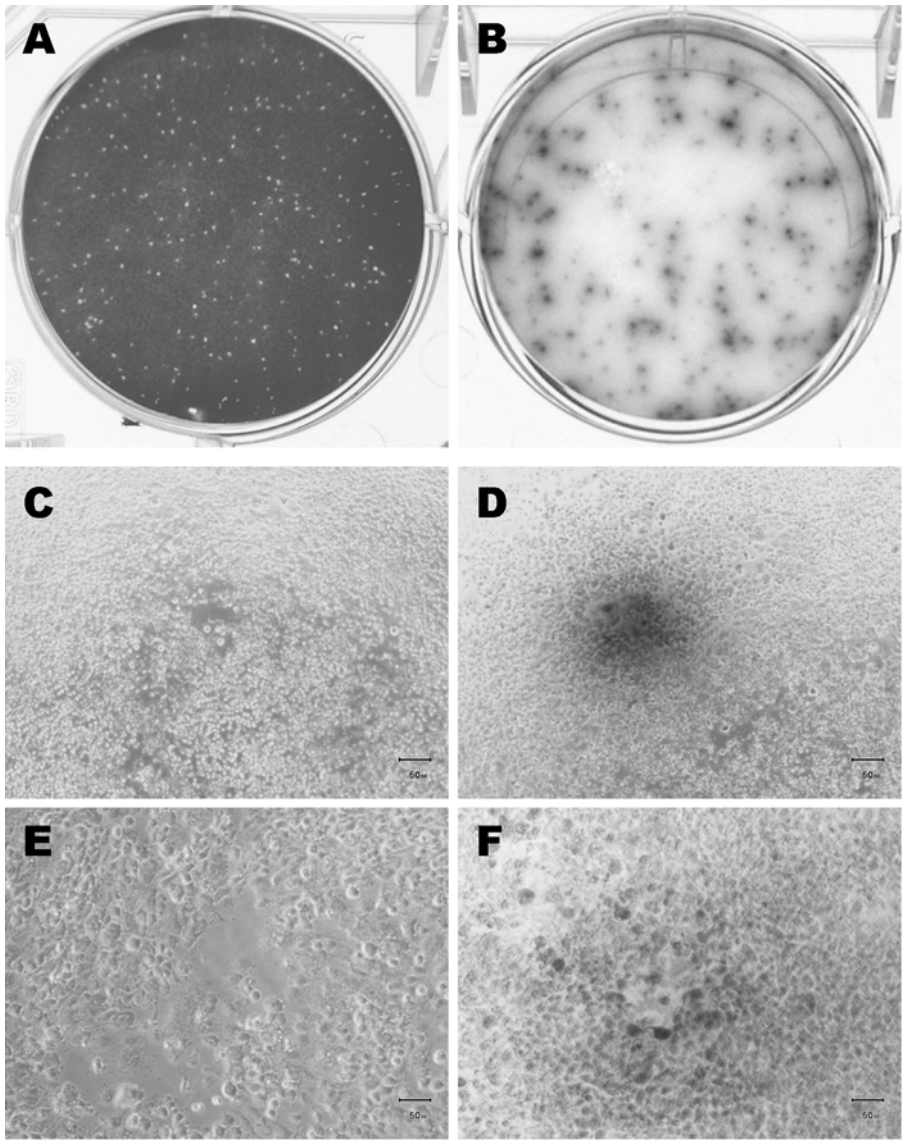

Fig. 2 Plaque formation of rMVA-VP2 and wild-type MVA in avian DF-1 cells. DF-1 cells were infected with 100 pfu/well of rMVA-VP2 (**b**, **d**, and **f**) or MVA-wt (**c** and **e**). 72 h post infection, titration was performed (**a**) or DMEM-0.6 % and Noble agar with X-Gal was added over the monolayer (**b**–**f**). *White plaques*: MVA-wt; *Dark plaques*: rMVA-VP2

6. Remove the medium and add complete DMEM–agar–X-Gal (*see* **Note 5**) over the monolayer.
7. Allow the overlay to solidify.
8. Incubate at 37 °C for 8 h.
9. Pick only well-separated blue plaques, about six per clone (*see* **Note 6**). To pick the plaques, insert the tip of the micropipette into the agar overlay just over the plaque, and draw the agar plug into the pipet. Transfer it into a small tube with 0.5 mL of complete DMEM and pipette up and down a few times to ensure that the plug does not remain in the pipette tip.
10. Carry out three cycles of thawing–freezing and sonication.
11. Repeat this cloning procedure (**steps 9** and **10**) about six times with each clone.
12. Amplify the cloned plaques using DF-1 cells.

3.3 Preparation and Titration of Virus Working Stocks

1. Passage DF-1 cells in 175 cm^2 flasks such they are confluent in 1 or 2 days.
2. Remove the old medium, add fresh medium and inoculate 0.1 MOI (*see* **Note 7**) of seed virus stock and Incubate DF-1 cells at 37 °C for 2–3 days until all cells show clear CPE; often, most cells will be floating.
3. Remove part of the medium (*see* **Note 8**), detach the cell monolayer and disrupt the cells with three cycles of thawing–freezing at –80 °C, transferring the medium and the disrupted cells to a new tube.
4. Sonicate in water bath the tube twice for 10 s, aliquot the rMVAs working stocks in volumes suitable for your purposes. We usually prepare aliquots of 1 mL each. Store at –80 °C.
5. For plaque assays of stocks prepare DF-1 cells in six-well plates 1 or 2 days prior to virus titration.
6. Thaw virus in 37 °C water bath, and make tenfold dilutions of the stock in complete DMEM. Each dilution must be mixed carefully and pipet tips changed between tubes (*see* **Note 4**). Transfer 100 μL of each dilution in each well.
7. Adsorb virus at 37 °C for 1.5 h, gently tilting back and forth every 15–20 min.
8. Aspirate the inoculum from higher to lower dilution wells, add 1.5 mL of complete DMEM from higher to lower dilution wells, and incubate for 3 days at 37 °C and air–5 % CO_2 atmosphere.
9. Fix the plates with 1 mL of 10 % formaldehyde for 30 min. Remove the medium and add 1 % crystal violet to stain the monolayers and count plaque numbers to calculate the virus titers (*see* **Note 9**).

3.4 Protein Expression Analysis

There are various methods to analyze the expression of the BTV proteins in DF-1 cells infected with the generated rMVAs. The two most common methods used in our laboratory are the immunofluorescence assay (*see* Fig. 3) and the immunoprecipitation of proteins in radiolabeled infected cells (*see* Fig. 4).

3.4.1 Immunofluorescence Assay

1. Plate DF-1 cells in 24-well plates with coverslips and incubate until they reach 80 % confluence.
2. Infect these cells with the rMVAs at an MOI of 1.
3. After 24 h of infection, fix the infected cells with acetone–methanol and store the plate at −20 °C for 20 min.
4. Remove acetone–methanol and wash once with 1 mL of PBS 1× (*see* **Note 10**).
5. Incubate the fixed cells with 1 mL blocking solution for 1 h.

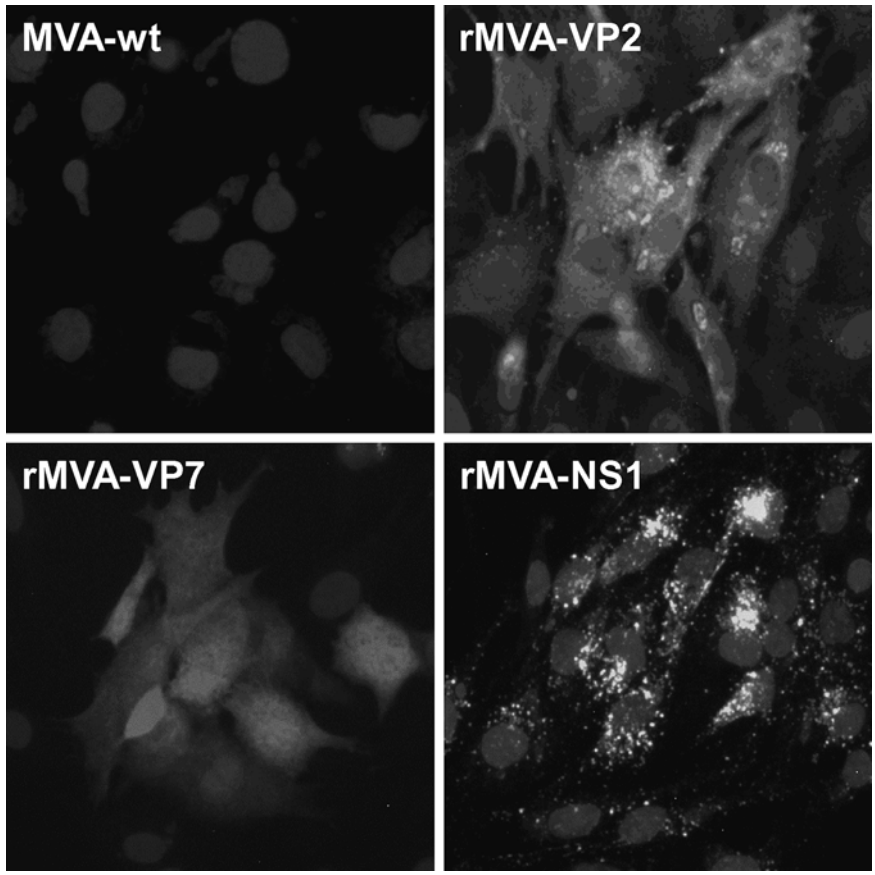

Fig. 3 Analysis of BTV-4 VP2, NS1, and VP7 expression by immunofluorescent staining. DF-1 cells were infected with recombinant MVA containing BTV-4 VP2, NS1, or VP7 genes. At 24 h.p.i, cells were fixed and analyzed by immunofluorescence by using a mouse polyclonal antisera specific of BTV-4

6. Remove the blocking solution and add the primary antibody. We use a mouse polyclonal antibody against BTV-4 diluted 1:500 or 1:1000 in blocking solution (it depends on each stock of sera) (*see* **Note 11**). We usually use 250 µL/well. Incubate at 4 °C o/n or room temperature for 3 h.
7. Remove the polyclonal antibody and wash with PBS 1× three times for 10 min preferably with shaking.
8. Add the conjugated secondary antibody (Alexa Fluor® 594 goat anti-mouse IgG (H + L)) specific of mouse primary antibody diluted 1:1000 in blocking solution over the cells. Incubate for 30 min at room temperature in dark conditions.
9. Remove the secondary antibody and wash with PBS 1× three times for 10 min with shaking preferably.
10. Mount the coverslips on slides using ProLong Gold antifade reagent and visualize using an immunofluorescence microscope.

3.4.2 Analysis of BTV Proteins Expression by Radiolabeling, Immunoprecipitation, and SDS-PAGE

Radiolabeling followed by immunoprecipitation is useful to analyze the expression of BTV proteins. VP2 protein contains conformational epitopes and polyclonal antibodies specific of BTV are not able to recognize the denatured protein by immunoblot. VP2, NS1, and VP7 can be immunoprecipitated with BTV-specific polyclonal antibodies from either BTV or MVA-VP2, MVA-NS1, and MVA-VP7 infected cells (*see* Fig. 4).

1. Infect DF-1 cells in 35-mm dishes with MVA-VP2, MVA-NS1, or MVA-VP7 at an MOI of 1.
2. After 90 min of virus adsorption, remove the culture medium and rise the cell monolayers with PBS 1× and once with methionine-deficient medium.
3. Add 1.5 mL of fresh methionine-deficient medium and incubate for 60 min (to starve of methionine).
4. At the end of starvation period, replace the medium and add medium containing [^{35}S] methionine (100 µCi/mL).
5. Incubate the cells for 16 h at 37 °C.
6. At the end of the incubation, remove the labeling medium and rinse the cells twice with PBS 1×.
7. Add 300 µL of RIPA buffer to each dish.
8. Leave the dishes on ice for 10 min.
9. Harvest the cell lysate to microfuge tubes. Vortex for 5 s and incubate on ice for another 10 min.
10. Centrifuge the tubes for 10 min at 8,050 × *g* to remove the cell debris and nuclei.
11. Transfer the supernatant to new microfuge tube and stand on ice or store at −20 °C.

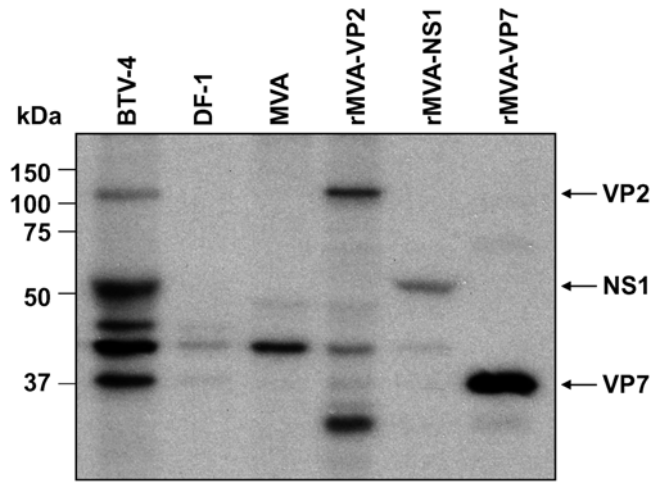

Fig. 4 Analysis of BTV protein expression by radiolabeling, immunoprecipitation, and SDS-PAGE. [^{35}S] methionine-labeled BTV proteins were isolated by immunoprecipitation using polyclonal antibody specific of BTV-4. The expression of BTV proteins in DF-1 cells infected with rMVA-VP2, rMVA-NS1, or rMVA-VP7 was then analyzed by SDS-PAGE

12. Immunoprecipitate BTV proteins with 10 μL of mouse polyclonal antibody specific of BTV-4 by using the Dynabeads® Protein G system and according to the protocol recommended by the manufacturer.
13. After the immunoprecipitation process, boil the beads in SDS-PAGE buffer for direct characterization of proteins on SDS-PAGE.

3.5 Purification of rMVAs by Using a Sucrose Gradient

There are various forms to purify and to separate viruses. Sucrose gradient is frequently used for separating virus, and the use of a sucrose cushion allows the possibility to concentrate the virus.

1. Layer 19 mL of the sonicated lysate onto 19 mL of a 36 % sucrose cushion (in PBS) in a sterile SW 28 (or SW 27) centrifuge tube (50 mL). Centrifuge for 90 min at 30,000 × *g* (SW 28 rotor) at 4 °C. Aspirate and discard the supernatant.
2. Resuspend the viral pellet in 0.5 mL of PBS 1× for a T150 flask (*see* **Note 12**).
3. Sonicate once for 1 min, and prepare a sterile 24–40 % continuous sucrose gradient in a sterile SW 27 centrifuge tube the day before it is needed by carefully layering 6.8 mL each of 40, 36, 32, 28, and 24 % sucrose. Let it sit overnight in the refrigerator.
4. Overlay the sucrose gradient with 1 mL of sonicated viral pellet and centrifuge for 50 min at 26,000 × *g* (11,500 × *g* an SW 27 rotor), 4 °C.

5. Observe the virus as a milky band near the middle of the tube. Aspirate the sucrose above the band and discard. Carefully collect the virus band (~10 mL) with a sterile pipet, place in a sterile tube, and save.
6. Collect aggregated virus from the pellet at the bottom of the sucrose gradient after aspirating the remaining sucrose from the tube. Resuspend the viral pellet by pipetting up and down in 1 mL of 1 mM Tris·Cl, pH 9.0.
7. Sonicate the resuspended pellet once for 1 min, reband the virus from the pellet as in **steps 5** and **6** and pool band with band from **step 6**. Add 2 volumes of 1 mM Tris·Cl, pH 9.0, and mix. Transfer to sterile SW 27 centrifuge tubes (*see* **Note 13**).
8. Centrifuge for 60 min at 32,900 × *g*, 4 °C, then aspirate and discard supernatant, resuspending the virus pellets in 1 mL of 1 mM Tris·Cl, pH 9.0. Sonicate as in last steps and divide into 200–250-μL aliquots. Store at −80 °C.

4 Notes

1. We found that 1 mL of TRI reagent is suitable for lysis of $5–10 \times 10^6$ cells.
2. Sequencing was performed by using a plasmid specific primer located 214 nucleotides downstream of the *Sma*I restriction site: *pSC11-A(VS): GTGGTGATTGTGACTAGCGTAG*.
3. The CPE caused after MVA infection consist of vacuolae formation spreading the cell cytoplasm. By using a Pipetman is easy to disrupt the monolayer, transferring the supernatants to a new tube to facilitate virus processing. It is important to use negative controls (infecting cells with MVA wt and MVA wt + Lipofectamine) and include a positive control (recombinant plasmid pSC11 + Lipofectamine).
4. We usually add 20 μL of the virus stock diluted in 180 μL of complete medium, and successively transfer 20 μL of the prior dilution to 180 μL of complete medium until dilution 10^{-7}).
5. In order to allow cell spreading, we use a proportion 1:1 between agar and medium. Do not try to do too many assays at the same time because the agar–DMEM mixture could solidify.
6. It is advisable to confirm the presence of the plaques by light microscopy.
7. In order to obtain a high viral titer is convenient to use low MOI to avoid a prompt damage in the cells.
8. We usually maintain 4 mL of medium/flask.

9. It is convenient to count the plaques of the intermediate dilutions, because it is easier to count (the size of MVA plaques are small).
10. In this step is possible to stop the procedure and the coverslips can be stored in PBS 1× at 4 °C for at least 1 month.
11. It is convenient to do tenfold dilutions of the sera in order to find the best dilution.
12. At this stage, the virus may be sufficiently pure for some purposes—e.g., isolation of DNA.
13. The total volume should be ~60 mL, which is enough to fill two SW 27 centrifuge tubes. If less volume is obtained, fill the tubes with 1 mM Tris–HCl, pH 9.0.

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REVIEW

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Defeating Bluetongue virus: new approaches in the development of multiserotype vaccines

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Bluetongue virus (BTV) is a global threat to domestic and wild ruminants, causing massive economic losses throughout the world. New serotypes of the virus are rapidly emerging in different continents, unfortunately there is little cross-protection between BTV serotypes. The eradication of the virus from a region is particularly complicated in areas where multiple serotypes circulate for a long time. The present review summarizes the actual concerns about the spread of the virus and relevant approaches to develop efficient vaccines against BTV, in particular those focused on a multiserotype design.

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KEYWORDS
• Bluetongue virus
• multiserotype protection
• vaccines • virus variability

Background

Bluetongue (BT) is a hemorrhagic viral disease that causes high morbidity and mortality in livestock species globally. The causative agent Bluetongue virus (BTV) belongs to *Reoviridae* family, genus *Orbivirus*, with a segmented dsRNA genome [1,2]. The virus is mainly spread between ruminant hosts through certain species of hematophagous *Culicoides* (Diptera, Ceratopogonidae) midges [3,4]. To date, 27 serotypes of BTV have been identified [5] and there are two further putative/novel BTV serotypes so far [6,7]).

The constant arrival of new BTV serotypes re-emphasizes the importance of making multiserotype and more effective vaccines than those that are currently available. Although conventional vaccines have controlled or limited BTV spreading in the past, they cannot address the need for cross-protection among serotypes. Moreover, live attenuated viral vaccines are associated with clinical signs, viremia level compatible with transmission and risk of gene segment reassortment; while whole inactivated vaccines are safer but less immunogenic and some serotypes of BTV do not replicate well *in vitro*. Strategies for Differentiating Infected from Vaccinated Animals (DIVA) would facilitate disease surveillance, especially in nonendemic countries. However, immunization of naive ruminant populations with either attenuated or inactivated vaccines against BTV precludes serological monitoring. This problem could be overcome using next-generation vaccines where only some genes or proteins of the virus are selected. Recent innovations in vaccine technology have been followed to generate a range of choices from subunit vaccines and recombinant viral vectors to reverse genetics based vaccines.

Viral structure & determinants of immunogenicity

The BTV genome consists of ten segments of linear dsRNA that encode seven structural proteins (VP1 to VP7) and five nonstructural proteins (NS1, NS2, NS3/NS3a, NS4 and NS5)

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(Table 1) [2,8–10]. The viral particle is a nonenveloped virion, approximately 90 nm in diameter, with an icosahedral capsid structure that packages the genome.

• Structural proteins

The genome is surrounded by copies of three minor proteins (VP1, VP4 and VP6), which are called transcriptase complexes, being involved in transcription and replication [11]. VP1 is the RNA-dependent RNA polymerase, VP4 acts as capping enzyme and VP6 works as RNA-dependent ATPase and helicase. After transcription of the genome segments in full-length mRNA copies, these are extruded from the core surface into the cytosol. Later on, VP1 also uses these mRNAs as a template for the production of new dsRNA segments, which are packaged within the subcore of the viral progeny. One copy of each genome segment is packaged per viral particle [12]. VP3 protein plays a crucial role in the virion assembly and is the component of the subcore [13]. This core internal layer serves as scaffold for the deposition of VP7 trimers, forming a stable core structure of 70 nm across. Proteins of the core, VP3 and VP7, present a high-sequence identity among different serotypes. VP7 is a major group-reactive antigen and a good stimulator of both the B-cell-mediated immune response and cytotoxic T-cell immunity [14,15]. Conserved $CD8^+$ and $CD4^+$ T-cell epitopes from VP7 amino acid sequence are present between serotypes [16,17].

The outer shell of the virion consists of VP2 and VP5 proteins, which are involved in cell penetration via endosomal route [18,19]. The BTV virion binds to the outer surface of susceptible cells through VP2 [20], while VP5 has a role in membrane destabilization [21]. The major component of the outer capsid VP2 is the most variable of BTV proteins and it is the main target of neutralizing antibodies [1,22–23]. It is known that VP5 enhances the neutralizing antibody response, mainly through its conformational influence on VP2 [1,24]. Differences within the outer capsid proteins of the virion are thought to reflect the influence of antibody selective pressure in the vertebrate host; in particular VP2 variability defines the identities of the identified BTV serotypes [7]. VP2 has been described as one of the most immunogenic protein, inducing both neutralizing antibodies and T-cell responses. Although these antibodies are serotype specific, sometimes antibodies raised against VP2 can partially neutralize other closely related serotypes [25,26].

• Nonstructural proteins

Nonstructural proteins are involved in either viral replication, assembly of new particles, egress or control of the innate immune response. The major expressed nonstructural protein NS1 multimerizes into cytoplasmic tubular structures in infected cells [27]. Previous works have elucidated that it might play some role in cellular

Table 1. Most relevant activities and immunological features of Bluetongue virus proteins.

Structural proteins	Segment	Main activities in viral life cycle and immunological features
VP1	1	RNA-dependent RNA polymerase
VP2	2	Cellular attachment. Viral-neutralizing antibodies
VP3	3	Assembly, RNA binding
VP4	4	Capping enzyme
VP5	6	Membrane destabilization. Enhance neutralizing antibody response
VP6	9	Helicase
VP7	7	Attachment to insect cells. Core antibodies. T-cell response
Nonstructural proteins	**Segment**	**Main activities in viral life cycle and immunological features**
NS1	5	Regulates protein translation. Form tubules in cytoplasm. CTL response
NS2	8	Inclusion bodies matrix. ssRNA binding. CTL response
NS3/NS3a	10	Viroporin. Interferes in IFN-I pathway
NS4	9	IFN-I antagonist
NS5	10	Unknown

CTL: Cytotoxic T cell; IFN: Interferon.

pathogenesis, possibly involving interactions with NS3/NS3a [28]. Other recent studies have indicated a role for NS1 in regulating viral protein translation [29]. Quimeric NS1 'tubules' displaying foreign epitopes on its surface can induce $CD4^+$Th cell and protective humoral responses [27]. In sheep, analysis of BTV-specific cytotoxic T cells (CTL) revealed VP2 and NS1 as major CTL targets [30]. Interestingly, sequences from NS1 identified as CTL epitopes are shared among a wide variety of BTV serotypes [31]. Expression of BTV NS2 in both insect and mammalian cells, results in the formation of viral inclusion bodies (VIBs). This ability together with the binding capacity to ssRNA suggests the role of NS2 in viral assembly, while VIBs are sites for BTV morphogenesis and RNA replication [32–34]. The only BTV glycoprotein NS3 has viroporin-like properties playing a role in virus release [35]. Furthermore, it is considered a determinant in viral pathogenicity, interfering with the IFN-I synthesis pathway [36]. There are two isoforms of NS3, both glycosylated: NS3 and NS3a; the latter lacking the N-terminal 13 amino acid residues [35,37–38]. NS4 protein has been recently identified, encoded from another open reading frame from segment 9, which encodes VP6 as well. This protein is highly conserved among several BTV serotypes/strains and seems to play a role in virus–host interaction. NS4 counteracts the antiviral response of the host as IFN type-I antagonist [39]. Very recently, a fifth nonstructural protein named NS5 has been identified from an alternative reading frame in segment 10. NS5 from transfected plasmids has the same cellular localization as BTV NS4 and has been speculated that both proteins may have a synergistic function [10].

Global expansion of BTV & variability

BTV is present in all continents, except Antarctica **(Figure 1)**, although BTV serotypes, topotypes and vector species differ in different regions of the world [40–43]. Among serotypes, VP2 shows 22.4–73% amino acid sequence variation [44]. To a lesser extent, VP5 is also highly variable between serotypes, with an amino acid identity of 41–79% among serotypes [45]. Therefore, the highest responsible of antigenic BTV variability is the outer capsid, while the core proteins, NS1 and NS2 are thought to be relatively conservative. NS3/NS3a are more variable than the other nonstructural or core proteins [46].

This vector-borne viral infection has undergone considerable expansion worldwide over the last decades, probably related with the global climate change and increase in international animal trade. As an example, since 1998 many BTV serotypes (serotypes 1, 2, 4, 6, 8, 9, 11 and 16) have invaded Europe [43,48]. First BTV was more limited to south Europe, when incursions affected only Mediterranean areas. However, circulation of different serotypes has increased in the last decade producing epizootics also in the North, reaching well beyond its known geographical upper limits. In 2006, BTV-8 emerged in central Europe and leads to an unprecedented epizootic causing serious problems to both animal health and economy [49,50]. Several previous exotic serotypes have appeared in Europe and all over the world, changing the already known distribution of the virus. This is the case of Australia and Israel, where new BTV serotypes have emerged recently [51–53]. Additionally, ten BTV serotypes have been detected since 1998 in the southeastern USA that were different to those previously found in North America [54,55].

BTV genome segments revealed variations between different strains belonging to the same serotype derived from distinct geographical regions [56–58]. It seems that individual BTV serotypes emerging in a region spend a period of geographic isolation, acquiring point mutations that lead to the generation of geographically distinct viruses called lineages or topotypes. Western and eastern topotypes are defined based on nucleotide sequence changes present in all of the BTV genome segments that partially correlate with the geographic origin of the virus [44,59–62]. The disease is more severe when an exotic BTV strain/topotype has incurred into a new region [58].

In order to restrict future outbreaks for the disease, it is essential to keep studying the molecular mechanisms promoting the change of BTV epidemiology. Genetic diversity of BTV has long been known to be influenced by segment reassortment and gene mutation [9,63–64]. The segmented nature of BTV genomic RNA can lead to exchange of genome segments in host cells infected with more than one BTV type [65,66]. It has also been suggested that intragenic recombination between strains can play a potential role in bringing new BTV lineages [67].

In the last 10 years, three novel confirmed and two further putative types of BTV have emerged in the different regions of the world [5,45,68].

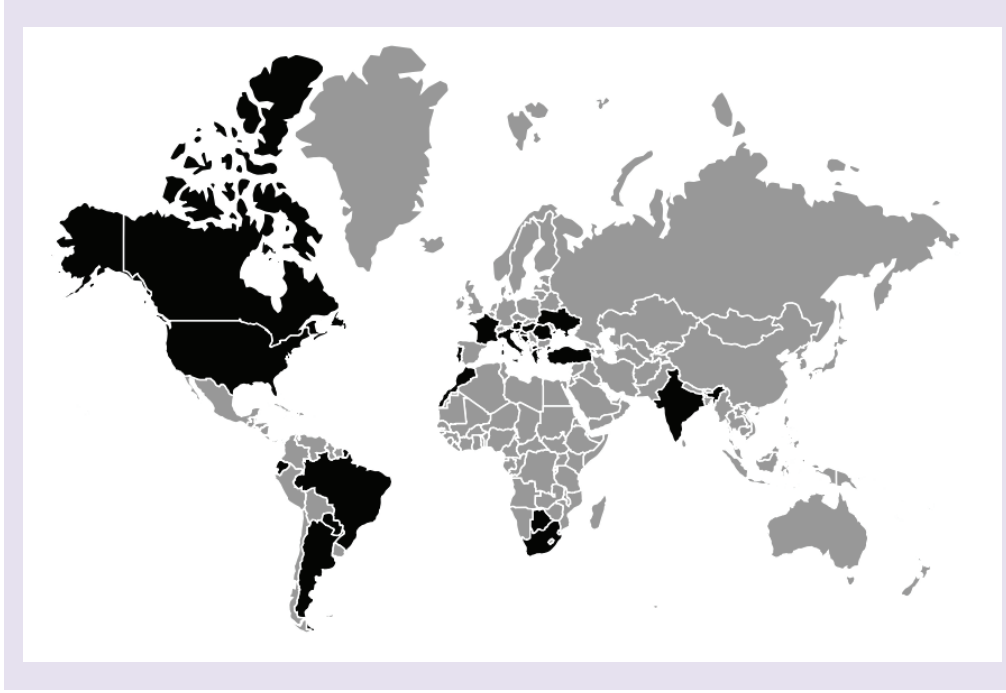

Figure 1. Information available from OIE-World Organization for Animal Health about the countries where Bluetongue virus has been present (in black) from July 2015 to May 2016. Reproduced from [47].

Along with the new serotype detected in goats in Corsica, France, currently 27 types of BTV have been described [5]. The emergence of new serotypes and the changes in global distribution exacerbate the problem to control BTV circulation and epidemics.

Seeking a multiserotype vaccine

• Classical vaccines

Prophylactic immunization of sheep and cattle is the most effective treatment to control BTV outbreaks, since there is no specific treatment for animals with Bluetongue. Vaccination is especially important to avoid expansion of BT endemic regions and to attempt to eradicate the virus. However, the use of live attenuated vaccines against BTV in the field does not fulfill the safety requirements. They can cause sufficient viremia to allow transmission that could lead to the origin of new strains of the virus through reassortment with circulating strains [69], making worse the scenario of BTV variability. As an example, a BTV-6 strain found in The Netherlands in 2008 contained segments from two other BTV strains circulating in the region [61]. Also, a BTV-16 vaccine strain containing Seg-5 from a BTV-2 strain used as vaccine was detected in Italy [69]. Although killed vaccines can be effective to limit the outbreaks, these do not induce a strong durable immune response and the use of some adjuvants produce severe adverse effects [70]. These conventional vaccines are usually not cross-protective against heterologous serotypes. Nevertheless, partial levels of protection against heterologous viruses have been described in some cases after immunization of ruminants with inactivated or modified live BTV vaccines **(Table 2)**. In a recent work, sheep inoculated with live attenuated BTV-4 were clinically protected against BTV-9 and BTV-11 challenges, however, they developed disease after infection with BTV-10 and BTV-1 [71]. Breard and coworkers [72] tested the potential protector effect of combinations of three inactivated commercial vaccines in sheep against virulent heterologous strains. Sheep first vaccinated with BTV-9, and later on with BTV-2 and 4, and challenged against BTV-16, showed a slightly reduced level of viremia and clinical score. As well, sheep immunized with two doses of BTV-9 were able to reduce viral loads in some animals after challenge with BTV-8. These results indicated that some partial protection against heterologous BTV is possibly afforded with killed or attenuated vaccines but it is highly dependent on the challenge serotype.

• Novel vaccine approaches

The novel vaccination strategies against BTV include subunit vaccines, recombinant viral vectors and vaccines based on reverse genetics. These vaccines have aimed at the induction of broadly neutralizing antibody and T-cell responses, since both arms of the adaptive immune response have a role in protection against BTV. Neutralizing humoral immune response rather than cellular immune responses are involved in protection against homologous serotype. However, cross-reactive T-cell responses against conserved antigens are needed to elicit protective immunity to heterologous BTV. VP2 is the main target of neutralizing antibodies, while NS1, VP7 and NS2 proteins have generally been described to induce cross-serotype helper T-cell or cytotoxic T-cell responses [30–31,73–74]. One of the greatest challenges for an effective vaccine against BTV is the virus variability, since new serotypes are regularly emerging. To try to overcome this obstacle, many vaccination strategies have been recently focused on targeting most conserved proteins of BTV in order to increase immune breadth **(Table 2)**.

Subunit vaccines for BTV have been mostly developed expressing single proteins or virus-like particles (VLPs) produced from recombinant baculoviruses or bacteria [75–77]. Only few of this kind of approaches have been targeted to heterologous BTV challenge viruses. Roy *et al.* studied the potential of VLPs (based on VP2, VP5, VP7 and VP3) to generate protective immune responses to heterologous serotypes [78]. VLPs from BTV-10 and BTV-17 origin were able to partially protect against BTV-4, triggering neutralizing antibodies against this related serotype (based on the amino acid sequence of VP2).

In a recent publication, baculovirus expressed nonstructural proteins (NS) NS1 and NS2 from BTV-2 have been investigated as vaccine in combination with VP2 from BTV-8 [79]. Low but specific T-cell proliferation responses were found against NS BTV-2 proteins, and also directed to UV-inactivated BTV-8, suggesting the cross-serotype reactions induced by these proteins. The authors suggested that inclusion of these NS proteins in vaccine strategies may facilitate the development of effective polyvalent BT vaccines. Since clinical and virological protection to BTV-8 was afforded in this study, further investigation is needed to determine the cross-protective capability of the vaccine against other serotypes [79].

Table 2. Summary of several vaccine approaches against heterologous Bluetongue virus challenge, describing immune responses and level of protection achieved.

Expression system	Proteins, serotype	nAbs to heterologous BTV	T-cell response	Challenge serotype	Clinical protection	Virological protection	Target species
Baculovirus	VLPs BTV-10 BTV-17	<log 0.6	NA	BTV-4	Partial	NA	Sheep
rgBTV-6	VP2, VP5 BTV-1	1:15–1:20	NA	BTV-8	Partial	Partial	Sheep
rgBTV-6	VP2, VP5 BTV-6	1:40–1:320	NA	BTV-8	Partial	Partial	Sheep
Inactivated	BTV-2 BTV-4 BTV-9	NA	NA	BTV-16	Partial	Partial	Sheep
Inactivated	BTV-2 BTV-4	NA	NA	BTV-8	NA	None	Sheep
Inactivated	BTV-9	NA	NA	BTV-8	NA	Partial	Sheep
DNA/MVA	VP2, VP7, NS1 BTV-4	<log 0.6	$CD8^+$ cells	BTV-8	Complete	Complete	Mice
DNA/MVA	VP2, VP7, NS1 BTV-4	<log 0.6	Na	BTV-8	Complete	Partial	Sheep
muNS-Mi inclusions	VP2, VP7, NS1 BTV-4	<log 0.6	$CD4^+$ high $CD8^+$ low	BTV-1	Partial	Partial	Mice

BTV: Bluetongue virus; MVA: Modified vaccinia virus Ankara; NA: Not analyzed; nAbs: Neutralizing-antibody response against heterologous serotype before challenge; NS: Nonstructural protein; rg: Reverse genetics; VLP: Virus-like particle.

In recent years, novel approaches against BTV based on reverse genetics strategies have emerged. The main strategy of these reverse genetic based vaccines is the generation of virions made up with proteins from different serotypes, in order to increase the range of immune response. One of the applications of this technology has been to develop disabled infectious single cycle vaccines (DISC) through the generation of replication-deficient BTV viruses [38]. The modified viruses carry a deletion in one or more genes that are essential for virus replication, such as the mutation in the gene encoding the helicase VP6 described in the work from Matsuo *et al.* [80]. This study showed that BTV-1 DISC reassortant virus with the genome segments 2 and 6 from BTV-8 prevented viremia after infection with BTV-1 or BTV-8. Additional works were made to produce DISC viruses from other serotypes (BTV-2, BTV-4 and BTV-8) and a combination of them was effective to protect sheep against clinical signs and viremia against the respective BTV types [81].

Reassortant BTV viruses were also developed based on the backbone of BTV-6 carrying the outer shell proteins VP2 and VP5 of BTV-1 (BTVac-1), BTV8 (BTVac-8) and BTV6 (BTVac-6) [82]. After infection with BTV8/net07 (homologous or heterologous challenge), vaccinated sheep had clearly reduced clinical reaction index, whereas all were negative for BTV8/net07. Although BTV6 regenerated by reverse genetics in this study was avirulent [36], after vaccination, the animals showed fever, mild clinical signs and were positive by the panBTV-PCR test for almost 2 weeks, reaching similar levels than unvaccinated controls when challenged. Further investigation will be useful to dismiss any potential bio-safety risk associated with the transmission in the field.

Another reverse genetic strategy is based on viruses missing *NS3/NS3a* coding gene [83]. These Disabled Infectious Single Animal (DISA) vaccines are made of live attenuated BTV as backbone and expressed several whole

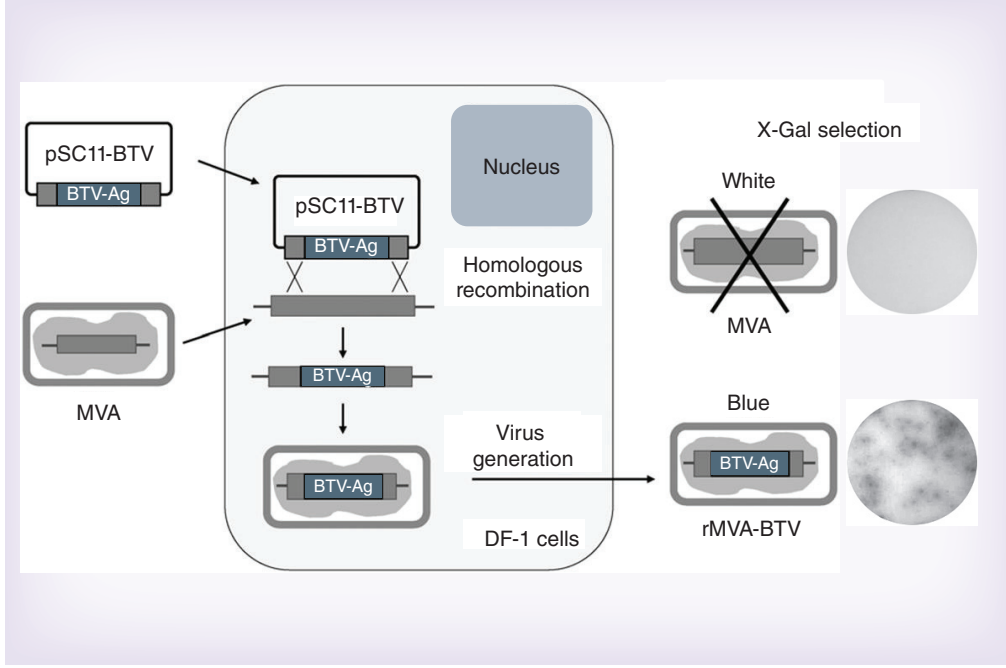

Figure 2. General procedure for the generation of recombinant modified vaccinia Ankara.
Genes encoding VP2, NS1 and VP7 of BTV-4 were cloned into the vaccinia transfer plasmid pSC11 downstream of the p7.5 vaccinia promoter. DF-1 cells were infected with modified vaccinia Ankara virus (multiplicity of infection 0.01 plaque-forming units/cell). After adsorption, cells were transfected with pSC11-VP2, pSC11-NS1, or pSC11-VP7 plasmids. Recombinant modified vaccinia Ankara viruses were generated by homologous recombination at the thymidine kinase locus, allowing the analysis by using the LacZ marker.
BTV: Bluetongue virus; MVA: Modified vaccinia Ankara; rMVA: Recombinant modified vaccinia Ankara.
Modified with permission from [98].

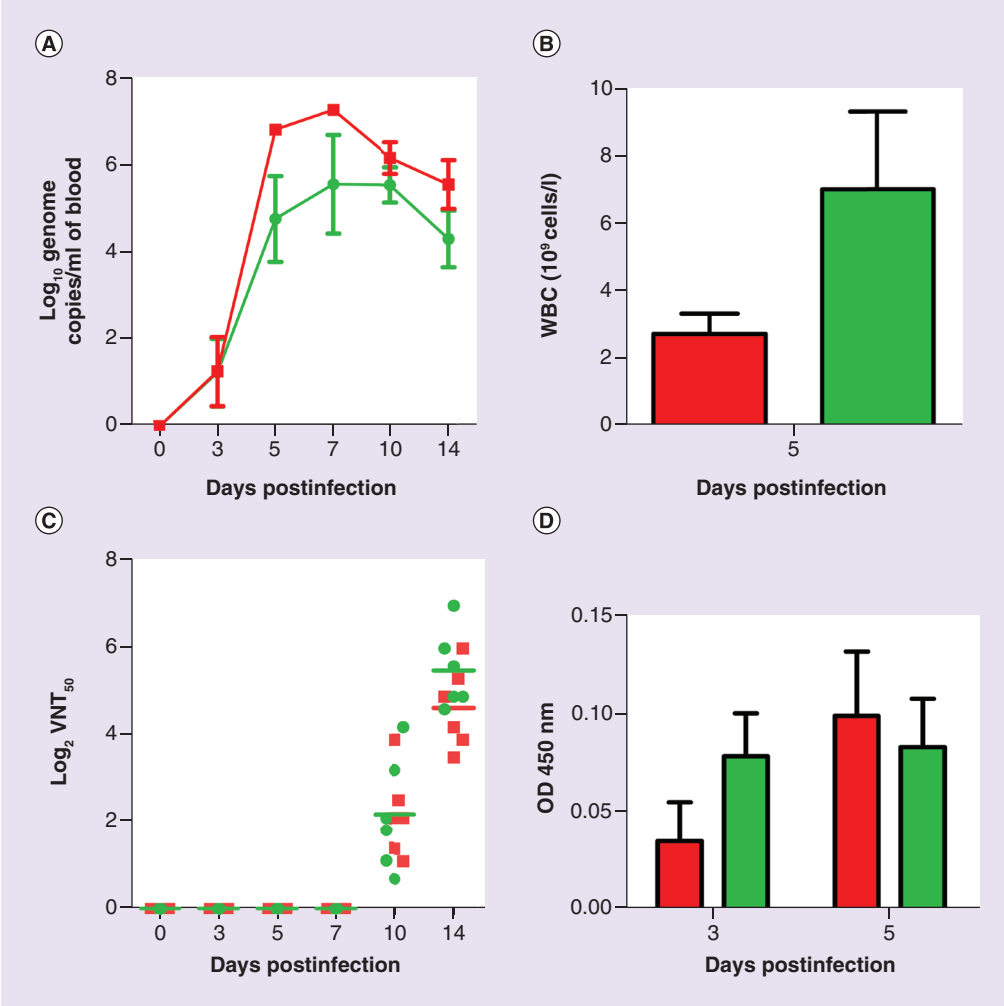

Figure 3. Study of cross-protection in sheep immunized with DNA/rMVA-VP2-VP7-NS1 after challenge with Bluetongue virus 8. Marroquina lambs (six per group) were immunized by heterologous prime boost vaccination with DNAs and recombinant modified vaccinia Ankara (rMVA)s expressing Bluetongue virus (BTV)-4 proteins (green) or pcDNA3 and MVA (nonimmunized lambs) (red), administered 3 weeks apart. A suspension of 100 μg of each pcDNA3 construct was administered intramuscularly and 10^7 plaque-forming units of each rMVA construct were inoculated subcutaneusly. Thirty-five days after the first immunization, lambs were challenged with BTV-8 to study cross-protection against the heterologous virus. **(A)** Number of amplified copies of BTV-8 genome in immunized and nonimmunized lambs after challenge. RNA was extracted from blood and viremia was determined by qRT-PCR. **(B)** Number of WBC determined by a semiautomated hematologic counter. Each bar represents the mean value of the WBC of six animals, and standard deviation are shown as error bars. **(C)** Titer of BTV-8 neutralizing antibodies in vaccinated and nonvaccinated lambs at different times postinfection. Values are based on the log2 of the highest dilution of the serum that reduced the number of viral plaques by 50%. **(D)** Level of IFN-γ measured in plasma after infection, determined by Bovine IFN-γ EASIA. Each column represents the average and the error bars show the standard deviation.
OD: Optical density; VNT: Viral neutralization test; WBC: White blood cell count.

or chimeric VP2 from other serotypes. Sheep vaccination with BTV1-DISA vaccine with 1/16 chimeric VP2 (150 aa from BTV-16) did not cause fever, clinical signs or viremia throughout the immunization trial and it developed specific neutralizing antibodies against both serotypes [84]. After the use of other DISA vaccine viruses, viremia was not detected using PCR

either. As well, knockout BTV-6 expressing BTV-8-VP2 was able to protect sheep against clinical signs and abolish viremia after challenge with virulent BTV-8 [85].

In another approach, inactivated BTV-1 has been used as backbone to express 220–429 aa from BTV-8 VP2. Conserved residues are expressed in the BTV-1 and BTV-8 VP2 junction sites, while divergent areas with neutralizing epitopes are also present. This chimeric virus was cross-neutralized by both BTV-1 and BTV-8 antisera, so the authors suggest the potential to develop constructs made by two or three synthetic strains with chimeric VP2 against multiple serotypes [86].

Among viral vectors used to develop BTV vaccine strategies, capripox, canarypox, adeno, herpes, myxoma, vesicular stomatitis and modified vaccinia Ankara (MVA)-based recombinant viruses have been tested [87–90].

A variety of viral vectored vaccines were generated expressing BTV major outer capsid protein VP2 (alone or in combination with VP5), with the aim to elicit a potent neutralizing antibody response capable to protect against same BTV serotype [91–93]. Immunogenic antigen VP7 is widely conserved across BTV serotypes and used in BTV serological diagnostic methods. This protein has been delivered by several viral vector vaccines mainly looking to increase cross-protection, since VP7 is a highly conserved protein and it is known to induce cell-mediated immune responses [16,17]. Of note, these strategies achieved different levels of partial protection, either against same or different serotype. A recombinant capripoxvirus expressing VP7 from BTV-1 serotype conferred partial protection against a heterologous BTV-3 infection in sheep [17]. In a more recent study, a nonreplicative canine adenovirus type 2 expressing VP7 from BTV-2 were evaluated against homologous or heterologous challenge (BTV-8). The immune responses triggered in sheep were not enough to afford protective immunity against BTV-8; however, partial protection was obtained against homologous challenge [88]. In a later study using adenovirus as gene delivery vector, VP7 expression alone or in combination with VP2 from BTV-8, displayed specific $CD4^+$ and $CD8^+$ T-cell responses

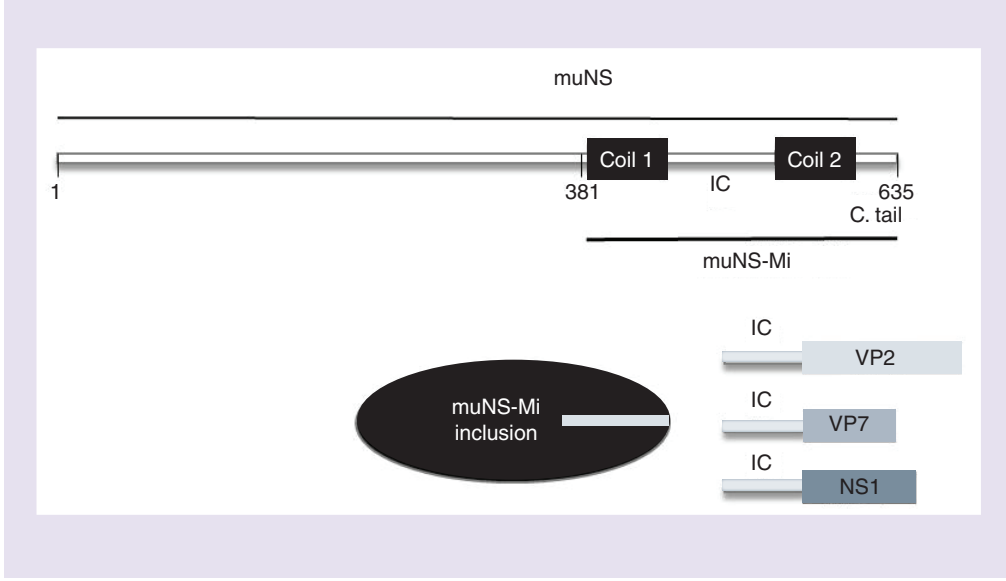

Figure 4. Construction of muNS-Mi microspheres tagged with Bluetongue virus antigens based on IC-tagging system. Avian reovirus (ARV) protein muNS is believed to form the matrix of viral factories because of its ability to form globular inclusions in the cytoplasm of infected cells. The minimal region able to form inclusions, muNS-Mi, (aminoacid 381–635) comprises a coiled-coil domain with two coil motifs separated by an IC and a C tail. As the IC domain is an interaction domain involved in muNS homo-oligomer formation, it causes relocation of the IC-tagged proteins (VP2, VP7 and NS1 from BTV-4) to ARV muNS-Mi intracellular globular inclusions, recruiting and concentrating the components of supramolecular complexes in the inclusions and increasing its efficiency as a vaccine.
IC: Intercoil sequence.

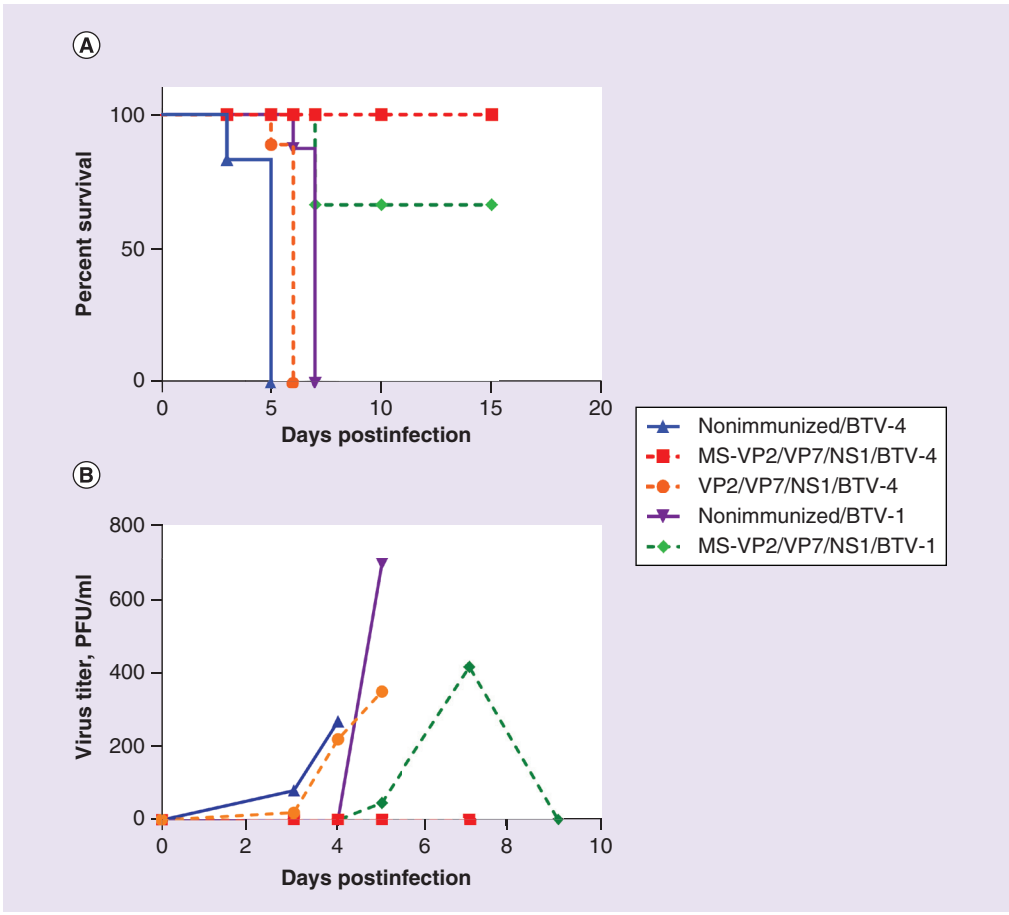

Figure 5. Protection of MS-VP2/MS-VP7/MS-NS1 vaccinated IFNAR -/- mice against Bluetongue virus 4 and Bluetongue virus 1 challenge. Mice (five per group) were immunized twice by homologous prime boost vaccination with recombinant VP2/VP7/NS1 soluble proteins or MS-VP2/MS-VP7/MS-NS1. Two weeks after immunization all mice were subcutaneously inoculated with 5×10^2 PFUs of BTV-4 or 10^2 PFUs of BTV-1. **(A)** Survival rates of immunized and nonimmunized mice after challenge. **(B)** Titers of BTV-4 and BTV-1 recovered in blood of immunized and nonimmunized mice after challenge. Virus was extracted from blood and viral load was determined by plaque assay. Each point represents the mean value of the group (modified with permission from [104]).
BTV: Bluetongue virus; PFU: Plaque-forming unit.

and neutralizing humoral response in mice and sheep. After challenge with BTV-8, all sheep developed viremia but at low levels than controls and the onset of viremia was delayed [87]. However, there was no evaluation of cross-protective immunity against heterologous BTV serotypes.

In our laboratory, several delivering systems as vaccine approaches against BTV have been developed, such as MVA, naked DNA and microspheres of muNS (virosomes from avian reovirus) tagged proteins. MVA is widely employed as vector vaccine for infectious diseases and has a well-established safety and efficacy record [94,95]. **Figure 2** describes the procedure for the generation of recombinant MVA. As well, several advantages as safety and easy manufacturing are linked to DNA vaccines. These are good inducers of immunity, especially to prime the immune system when used in heterologous vaccination regimes [96]. On the other hand, muNS methodology allows to create multiepitope particulate material for immunization purposes that could have potential advantages as vaccines [97].

Transgenic mice deficient in type-I interferon (IFN) receptor (129sv IFNAR-/-) mice support the growth of BTV *in vivo* and represent a small animal model useful for the study of pathogenesis and potential vaccines against BTV [99]. Initial vaccination studies in IFNAR-/- mice with recombinant vectors DNA and MVA expressing

VP2, VP5 and VP7 proteins (DNA/MVA-VP2-VP5-VP7) from BTV-4, showed the absence of clinical signs of disease or viremia at any point after infection with lethal BTV-4 challenge [100]. Nevertheless, when this vaccine was evaluated against the heterologous serotype BTV-8, just partial heterotypic immunity was conferred, showing a delay of the disease and onset of viremia. Due to the importance of CTL response in cross-protection against BTV [14], VP5 was exchanged by NS1 in the composition of the vaccine. NS1 is an immunogenic protein, highly conserved between serotypes and major CTL target in sheep and mice [30,31]. Vaccination of IFNAR-/- mice with DNA/MVA-VP2-VP7-NS1 from BTV-4 in a prime boost regime generates a strong neutralizing antibody response against BTV-4 and stimulates specific $CD8^+$ T-cell responses. Recombinant VP2, VP7 and NS1 proteins *in vitro* induced the expression of specific IFN-γ by $CD8^+$ T cells from vaccinated mice upon restimulation. In addition, immunization displayed increased levels of several cytokines, such as IL-12, IL-1β and IL-6 in serum. After heterologous challenges, animals immunized with this vaccine approach were completely protected against lethal challenge with BTV-1 and BTV-8 [101]. No viremia was detected at any time of the experiment. By contrast, nonvaccinated mice died after viral infection between day 5 and 7 days postinfection. Interestingly, this recombinant vaccine cross-protected against no phylogenetically close serotypes. Further studies were carried on in sheep in order to evaluate the level of heterotypic cross-protection against distinct BTV by this approach in a natural host [102]. When challenged with BTV-8, animals vaccinated with DNA/MVA-VP2-VP7-NS1 (BTV-4 origin) exhibited lower levels of viremia than nonimmunized animals **(Figure 3)**. Leukopenia was observed in nonimmunized lambs, whereas vaccinated lambs maintained normal levels (7×10^9cells/l) of white blood cell count (WBC). Lowest level of WBC in control sheep was detected when viral load in blood was higher, which correlate with findings in previous BTV pathogenesis studies [103]. Results from this experiment in sheep revealed partial heterotypic protection achieved by the vaccine candidate to phylogenetic unrelated serotype.

Another followed approach is based on the IC-tagging methodology, a tagging and inclusion-targeting system based on muNS virosomes from avian reovirus. The method is based on immobilization of proteins in muNS-Mi inclusions (microspheres or MS) in baculovirus-infected cells **(Figure 4)**. The MS can simultaneously recruit several tagged proteins [97] generating multiepitope complexes. Recombinant VP2, VP7 and NS1 proteins from BTV-4 were tagged to MS with immunization purposes (MS-VP2/MS-VP7/MS-NS1). Vaccination of IFNAR -/- mice with this subunit vaccine (without adjuvants) evoked specific $CD4^+$ and $CD8^+$ T-cell responses against BTV antigens [104]. When mice were challenged against a homologous BTV-4, immunization conferred full protection against clinical signs and viremia. In immunized animals infected with heterologous BTV-1, vaccine efficacy approached 60%, indicating that partially cross-protected against a phylogenetically distant BTV was achieved **(Figure 5)**. Since T-cell response triggered by MS-VP2/MS-VP7/MS-NS1 was mainly $CD4^+$, the development of smaller MS that could elicit higher $CD8^+$ T-cell response is suggested, with the aim to improve cross-protection against multiple BTV serotypes.

Conclusion & future perspective

Inactivated, live attenuated, recombinant antigens, DNA, viral vectored vaccines, as well as reverse genetics technology, have been employed to pursue protective vaccines against BTV. Relevant studies on BTV immunology have highlighted the most important viral determinants triggering immune responses that enhance cross-protection among BTV serotypes. CTL against conserved epitopes are most likely to enhance the breadth of the immune response against multiple serotypes. However, most already described vaccines are not fully effective against distinct BTV and the major obstacle currently to control BTV is the genetic variability of viral isolates worldwide. Therefore, efforts are still needed to devise immunization strategies to increased heterotypic immune responses in the vaccine candidates against a broad range of BTV serotypes. In the light of new technologies and the continuous advances in this field of research, BTV vaccine development will hopefully progress to fit the current epidemiological requirements.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

EXECUTIVE SUMMARY

Bluetongue virus: a global concern

- Bluetongue virus (BTV) is a widespread vector-borne pathogen that can cause severe hemorrhagic disease in ruminants.
- The constant arrival of new BTV serotypes re-emphasize the importance of making multiserotype vaccines.

Viral structure & determinants of immunogenicity

- VP2 protein induces both neutralizing antibodies and T-cell responses.
- Neutralizing antibodies are serotype specific in general, some antibodies raised against VP2 can sometimes neutralize less efficiently other closely related serotypes.
- NS1, VP7 and NS2 proteins have generally been described to induce cross-serotype helper T-cell or cytotoxic T-cell responses.

Global expansion of BTV & variability

- Currently 27 types of BTV have been identified.
- The emergence of new serotypes and the changes in global distribution exacerbate the problem to control BTV circulation and epidemics.

Seeking a multiserotype vaccine

- Conventional vaccines against BTV in the field do not fulfill current requirements.
- The novel vaccination strategies against BTV include subunit vaccines, recombinant viral vectors and vaccines based on reverse genetics.
- Multiserotype strategies are focused on targeting most conserved proteins of BTV in order to increase immune breadth.

Conclusion & future perspective

- The major obstacle currently to control BTV is the genetic variability of viral isolates worldwide.
- Efforts are still needed to devise immunization strategies to increased heterotypic immune responses in the vaccine candidates against a broad range of BTV serotypes.

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